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EXPERIMENTAL ROENTGEN INJURY. V. EFFECTS ON HEMATOPOIETIC RESERVES AND REGENERATIVE CAPACITY

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INTRODUCTION

In previous studies (*1–3*), a systematic investigation was made of the changes produced in the blood and hematopoietic organs of mice following single whole-body exposures to different-sized doses of X-rays. This work revealed injury in the form of karyorrhexis and karyolysis in the spleen, lymph nodes, and bone marrow. The damage was observed as early as 2 hours after treatments with 100 to 400 r and increased in amount up to 12 hours. Phagocytic cells with engulfed nuclear debris increased in number during the period 6 to 12 hours, but after 24 hours most of the tissue debris had disappeared. Depressed total leukocyte levels were also observed, which lasted 3 to 6 weeks or more. When the doses were larger, the tissue destruction was greater; symptoms of shock developed, and blood dyscrasias appeared and persisted longer. When the doses were less, the signs of injury were less apparent, and a dosage level (50-r single exposure) was found below which there was no observable variation from the normal. The question arose whether some degree of injury might not be produced by the lower dosages but might be undetectable by the methods of observation used.

In still another study (*4*), repeated small exposures (5, 10, 15, 20, and 25 r per exposure) were given five times weekly. Single treatments of these amounts failed to elicit detectable signs of injury; but after a series of applications, changes were manifested in all animals, which resembled in many ways those obtained with the larger single doses. Considerable interest attaches to these changes as they appear to involve the various tissue reserves. Since the changes produced by single applications of a small dose cannot be seen by the use of methods now available, they may be regarded as subliminal. The present experiments were designed to deal with subliminal effects and with the reserve capacities of certain tissues.

EXPERIMENTAL PROCEDURE

Strain C3H male mice were used exclusively. The animals were 2 to 3 months old when the experiment started, the actual age of each animal at the time of treatment being known precisely. Forty animals were used for each experimental sample, and single acute doses were applied to the whole body.

In an earlier experiment (*1*) in which single doses of 50 and 100 r were given, only mild changes in the tissues and in the peripheral blood were produced. Hence repeated administration of such doses might be expected to give an ap-

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preciable amount of change in a few weeks. In the present work, in addition to 50- and 100-r doses, the following were given: 12½, 25, 200, and 400 r per treatment. The 12½-r dosages were administered twice weekly (Wednesday and Saturday), the 25-r once weekly (Saturday), and the 50-r at fortnightly intervals (alternate Saturdays). Some sequence of repeat treatments was necessary for the dosage used, as one treatment was not sufficient to lower the blood level to the point desired.

The lymphocyte level was used as a guide. Since this level in normal C3H mice is usually 5,000 to 10,000 cells per cubic millimeter of whole blood, 4,000 was arbitrarily chosen as the lower limit of normal. Treatments were given until the lymphocytes dropped significantly below 4,000, at which time they were discontinued until the lymphocyte level rose above 4,000 for 2 consecutive weekly counts. By this procedure, it was expected that a pattern of treatment might be obtained, the results of which would indicate the rapidity of accumulation of certain effects and also the rapidity of recovery at different times after treatment. By comparing the cycles of injury and repair at different times, it was believed that information could be obtained about the reserve capacities of hematopoietic organs.

Regular erythrocyte counts were not made, as previous experience had shown red-blood-cell levels to be little affected by radiation (except terminally). The previous experience was borne out in the present work by occasional counts.

Total leukocyte and differential counts were made at fortnightly intervals on six animals from each dosage group. Instead of making counts on all six at one time, counts were made on three one week, and three the next. This procedure gave weekly counts but did not require the same mouse to be bled more often than once in 2 weeks. In each case the counts were averaged and the values plotted (figs. 1 and 2). The differential leukocyte counts included eosinophils, basophils, monocytes, myelocytes, and blast forms, as well as neutrophils and lymphocytes. Thus, in the graphs where the sum of the neutrophils and lymphocytes does not equal the total leukocyte count, the difference may be attributed to the other forms. No further mention will be made of the other forms, however, as the data showed little of interest in the present considerations.

RESULTS

HEMATOLOGIC FINDINGS

The results of the treatment indicated in figures 1 and 2 cover a period of approximately 1 year after treatment began.

The graph for controls (fig. 1) shows that the normal leukocyte level fluctuated mainly between 10,000 and 20,000; only occasionally did it go above or below this range. Likewise the curves for neutrophils (dotted) and lymphocytes (dashed) show that these cells varied generally within the limits of 5,000 and 10,000.[1] The lymphocyte level fell below 4,000 on only 3 occasions, and only once (at 32–33 weeks) for as long as 2 consecutive weeks. This fact fully justified the decision at the beginning to consider 4,000 as the normal lower limit for lymphocytes.

No graph for the animals receiving 12½ r per treatment is included, as there was nothing in the graph for this group that is not better shown in the other graphs.

The graph for animals receiving 25 r per treatment shows, as do all the others, that 2 blood examinations were made 1 week apart just before the first treatment was given. After this treatment, the lymphocyte number rose, but not beyond the normal range. This rise was followed by a steady decline, until at 5 weeks, when the sixth treatment was given, the number was only slightly above 2,000. Treatment was then discontinued, and the lymphocyte level rose

[1] Usually in C3H control mice the proportion of lymphocytes is somewhat greater, but in this particular group it remained about the same as that of the neutrophils.

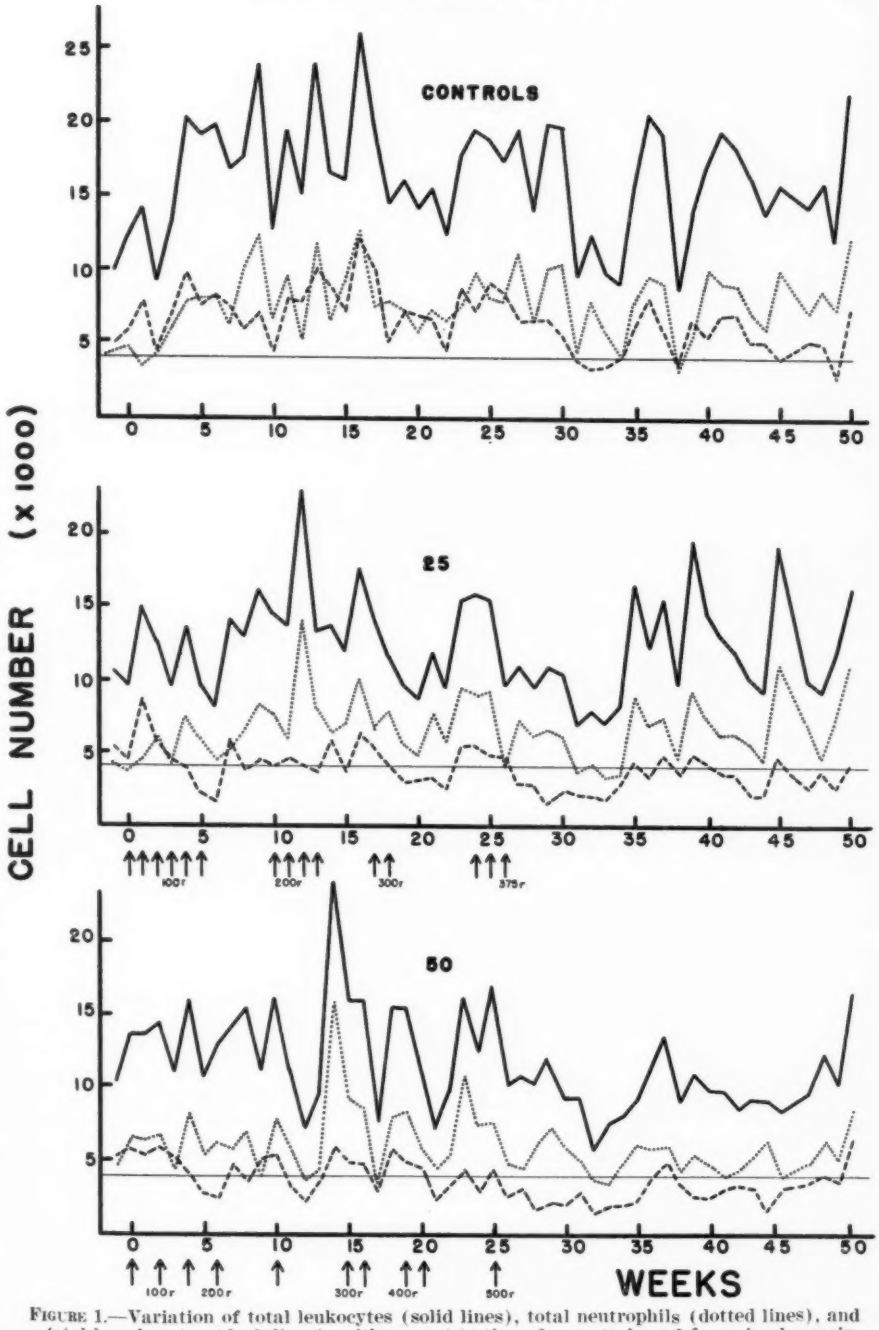

FIGURE 1.—Variation of total leukocytes (solid lines), total neutrophils (dotted lines), and total lymphocytes (dash lines), with respect to time, for controls and for animals receiving the 25- and 50-r treatments. The arrows below each graph show the times when treatments were given, the small figures by the arrows give the accumulated doses at different times, and the fine horizontal lines indicate the level of 4,000 cells.

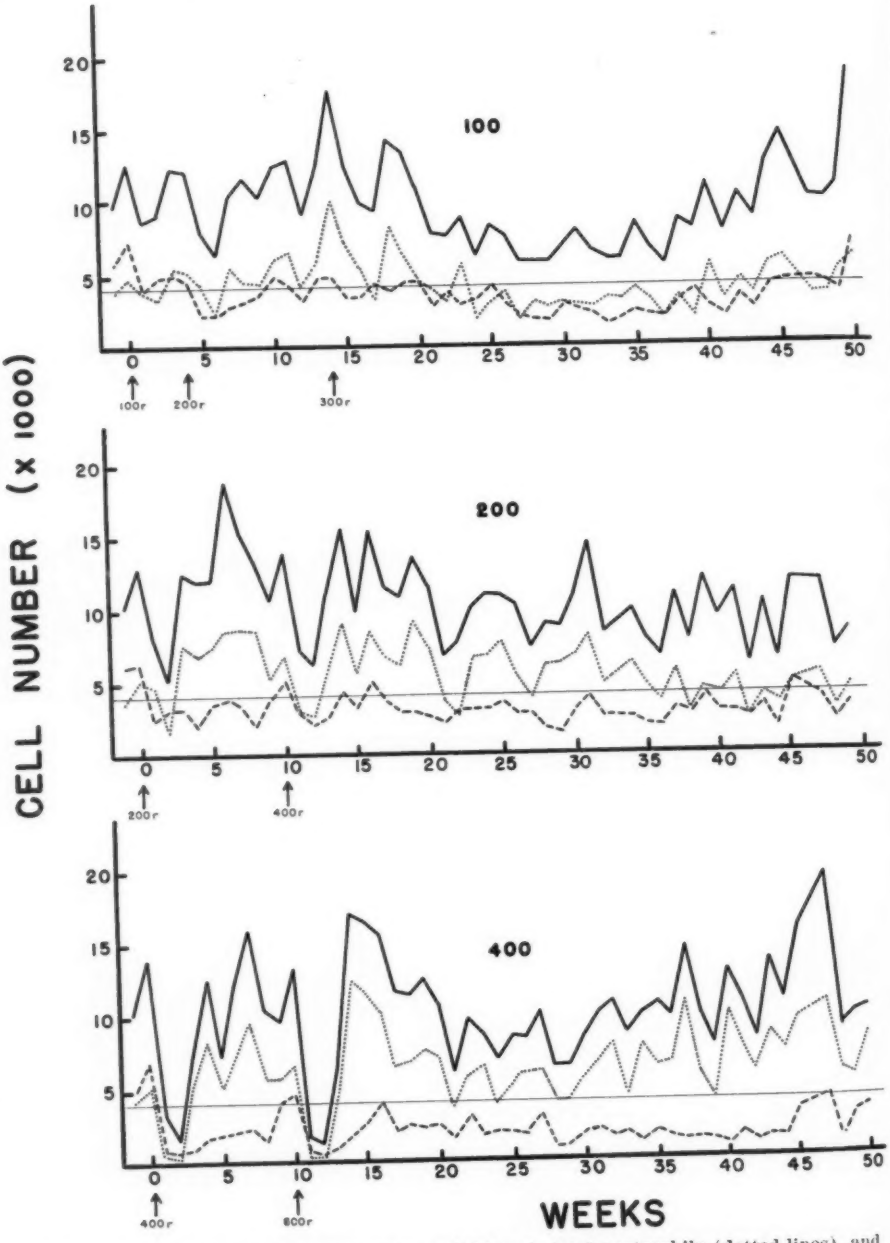

FIGURE 2.—Variation of total leukocytes (solid lines), total neutrophils (dotted lines), and total lymphocytes (dash lines), with respect to time, for animals receiving 100, 200, or 400 r per treatment. The arrows below each graph show the times when treatments were given, the small figures by the arrows give the accumulated doses at different times, and the fine horizontal lines indicate the level of 4,000 cells.

to 6,000 within 2 weeks. The number then dropped to less than 4,000 during the next week. At the ninth and tenth weeks, counts of 4,500 and 4,000, respectively, were obtained. Hence, the treatment was resumed. After the fourth treatment of this second cycle, the leukocyte level dropped to less than 4,000 so that treatment was stopped. During the interval that followed, the leukocyte level fluctuated between 4,000 and 6,000, and it was not until the seventeenth and eighteenth weeks that 2 consecutive counts above 4,000 were obtained. After 2 more treatments, the lymphocyte level fell below 4,000 where it remained for some time. However, the level rose again, and 2 consecutive counts above 4,000 were obtained at the twenty-third and twenty-fourth weeks. After 3 further weekly applications, the level fell below 4,000 and did not show 2 consecutive counts above that level during the remainder of the experiment, which covered a period up to 50 weeks. The total accumulated dosage was 375 r. Following the administration of this amount of radiation, the animals tended to show a persistently lower lymphocyte level. It is plain that the number of treatments required to bring the lymphocyte level below 4,000 diminished as the experiment progressed, and that the period required for recovery grew longer.

The graph for animals receiving 50 r per treatment reveals the same general characteristics as observed for those receiving 25 r. The total dosage was 500 r, but the same tendency towards a permanently lowered lymphocyte level was perhaps more striking. Much the same treatment and recovery patterns were also manifested.

The graph for animals receiving 100 r per treatment shows even more remarkable changes. The lymphocyte level following the first exposure dropped abruptly to 4,000, and recovery was slow. After a second 100-r treatment at 4 weeks, there was a second abrupt fall in lymphocyte level to less than 2,500. Recovery in this instance was extremely slow, and it was not until the thirteenth and fourteenth weeks that 2 consecutive counts above 4,000 were obtained. After a third 100-r treatment at the fourteenth week, the lymphocyte level tended to remain in the lower range for the remainder of the experimental period. The total accumulation of radiation in this case was 300 r. The tendency toward a permanently lowered level set in at an earlier period, and the interval between the second and third treatments was distinctly longer than that between the first and second. In this series, the neutrophils also tended to show a permanently lowered level and followed along in a range about the same as the lymphocytes.

The graph for animals receiving 200 r per treatment (fig. 2) shows still more abrupt changes. After the first application, there was a marked fall in both the neutrophil and lymphocyte levels; the neutrophils showed a quick return to normal, but 2 consecutive counts above 4,000 for the lymphocytes were not obtained until the ninth and tenth weeks, at which time a second treatment was given. Again there was an abrupt fall of both neutrophils and lymphocytes; and again there was a quick return to the normal range in the case of neutrophils, but the lymphocytes did not show 2 consecutive counts above 4,000 during the remainder of the experimental period. Here the total accumulation of dosage was 400 r, but the larger increments of dosage prevented as fine a determination of dosage at the time the animals first reached the permanently lowered lymphocyte condition. It appears from the graph that a single dose of 200 r was nearly sufficient to put the animals into the permanently lowered lymphocyte condition, but according to the plan of the experiment it was necessary to repeat the treatment at the tenth week. Whereas 2 consecutive counts above 4,000 were seen within 10 weeks after the first treatment, no corresponding 2 counts were seen at any period after the second treatment.

The graph for animals receiving 400 r per treatment portrays the most strik-

ing changes of all. After the first treatment, both the neutrophil and lymphocyte levels dropped to less than 1,000 within 1 week. They remained low during the second week, but during the third week recovery, especially in the neutrophils, began. Recovery of the lymphocytes was slow, but 2 consecutive counts above 4,000 were obtained at the ninth and tenth weeks, at which time a second treatment was administered. Again there was an abrupt fall of both lymphocytes and neutrophils to less than 1,000 and again these levels remained less than 1,000 for a period of 1 week. Recovery was extensive in the case of neutrophils but slow in case of the lymphocytes. The lymphocytes in fact did not go above 4,000 at any time during the remainder of the experiment. It seems likely that the lymphocyte level for the most part would have remained below 4,000 even if the second treatment of 400 r had not been applied.

In summary, the general trends noted in connection with the group receiving 25 r per treatment were borne out by the groups receiving higher treatment increments. In general, the 100-r dose appears to have been the most satisfactory for demonstrating the various features. The first application of this dose was sufficient to lower the lymphocyte level directly to 4,000, but not beyond. Following this dose, recovery was rapid. Reapplication of the 100-r dose, while the lymphocytes were in the lower range of normality, dropped the lymphocytes to well below the 4,000 level. After this, recovery was considerably slower, and the interval between the second and third treatments was much longer than between the first and second. From an examination of the graphs, it appears that three 100-r doses are very nearly the lower limit of dosage that can be used in this manner to obtain persisting lymphopenia. The graphs for animals receiving 200 and 400 r show certain additional features: The neutrophils responded as readily and as extensively as the lymphocytes, and recovery took place in both cases, although more abruptly and more extensively in the neutrophils.

HISTOPATHOLOGIC FINDINGS

In addition to the hematologic study, a histologic study was made. Two animals from each experimental group were killed as a rule at each of the following times after the beginning of treatment: 1, 2, and 3 weeks, and 1, 2, 3, 5, 7, and 11 months.

Generally, a direct correlation could be made between the peripheral blood picture and the condition of the hematopoietic tissues. Figure 3, *A*, shows a section of a node at 6 weeks that had received four 50-r treatments (the treatments being given at intervals of 2 weeks). In contrast with normal lymph nodes, which usually show numerous areas of small, deep-staining lymphocytes, this node shows only a few of these cells. The gland consists largely of reticular elements and shows a slight relative increase in fibrosis. Figures 3, *B*, and 4, *A*, show similar nodes also at 6 weeks which had received, respectively, two 100-r treatments 4 weeks apart and one 200-r treatment at the beginning. About the same degree of damage was produced in all three nodes irrespective of whether the doses were given all at once or at intervals. Figure 4, *B*, shows a lymph node at 4 months after receiving two 200-r treatments 10 weeks apart (i. e., 4 weeks after the second treatment). Here there was even greater loss of lymphocytes with perhaps a greater relative increase in fibrosis.

Figures 5 and 6 show changes in bone marrow following a single 400-r dose. Figure 5, *A*, indicates the condition at 1 week after treatment when the marrow showed an extreme degree of acellularity and the blood sinuses occupied most of the space. This was the picture when the total leukocyte level was in the neighborhood of 2,000 to 4,000 cells, and the neutrophil and lymphocyte counts below 1,000. At 2 weeks, when the lymphocyte level was still very low, the bone marrow showed signs of slight regeneration (fig. 5, *B*), and at 5 weeks it had reached a stage almost indistinguishable from the normal (fig. 6, *B*). At this stage, i. e., 5 weeks, the neutrophils had returned to the normal range,

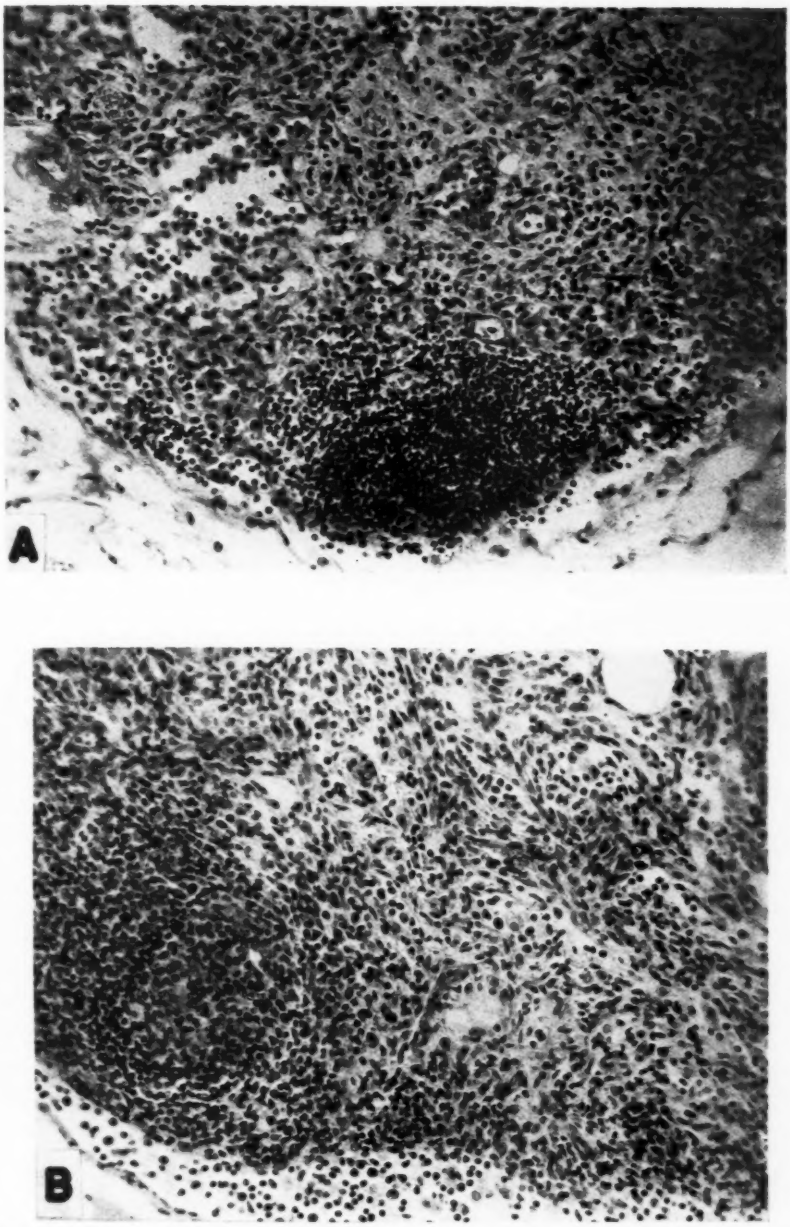

FIGURE 3.—Lymph nodes. *A*. From animal receiving four 50-r treatments 2 weeks apart, tissue fixed at 6 weeks after first treatment; *B*. From animal receiving two 100-r treatments 4 weeks apart, tissue also fixed at 6 weeks. Hematoxylin and eosin stain. × 335.

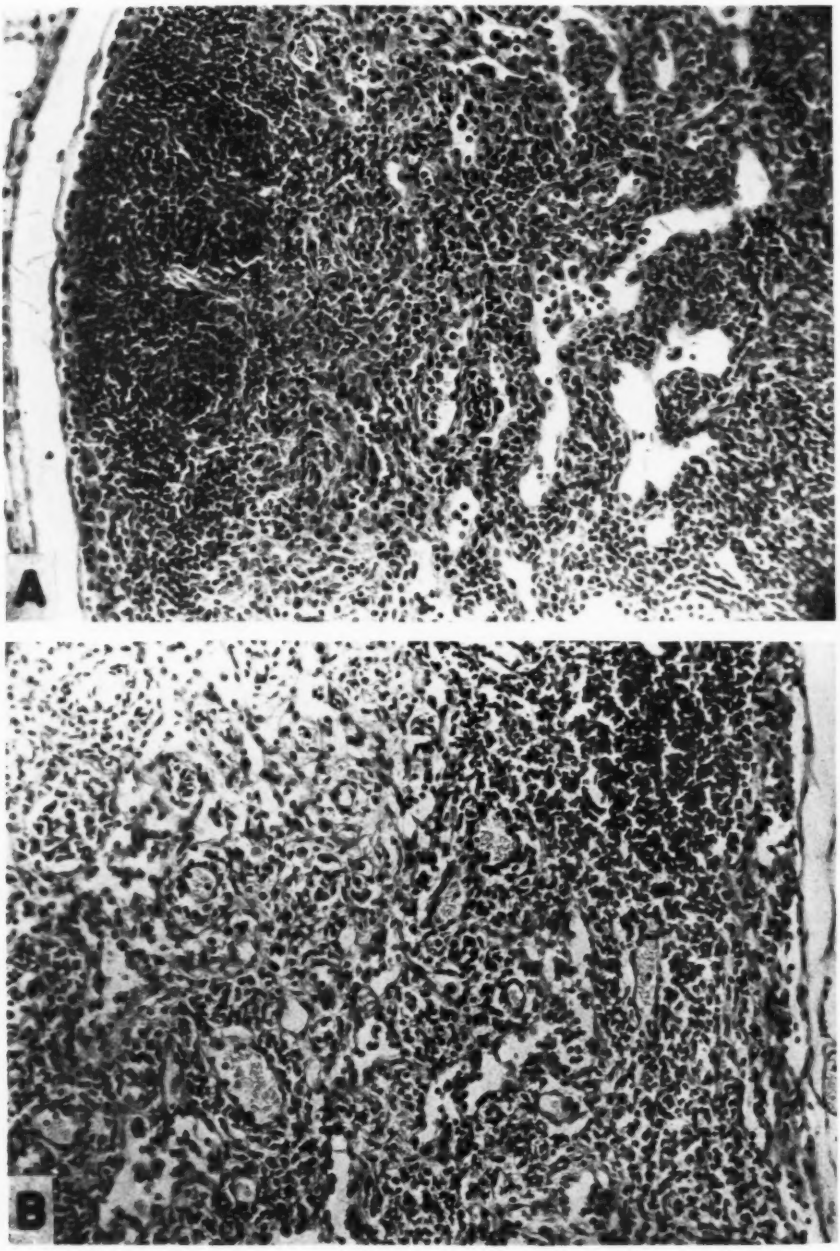

FIGURE 4.—Lymph nodes. *A*, From animal receiving one 200-r treatment, tissue fixed at 6 weeks after first treatment; *B*, From animal receiving two 200-r treatments 10 weeks apart, tissue fixed at 4 months after first treatment. Hematoxylin and eosin stain. × 335.

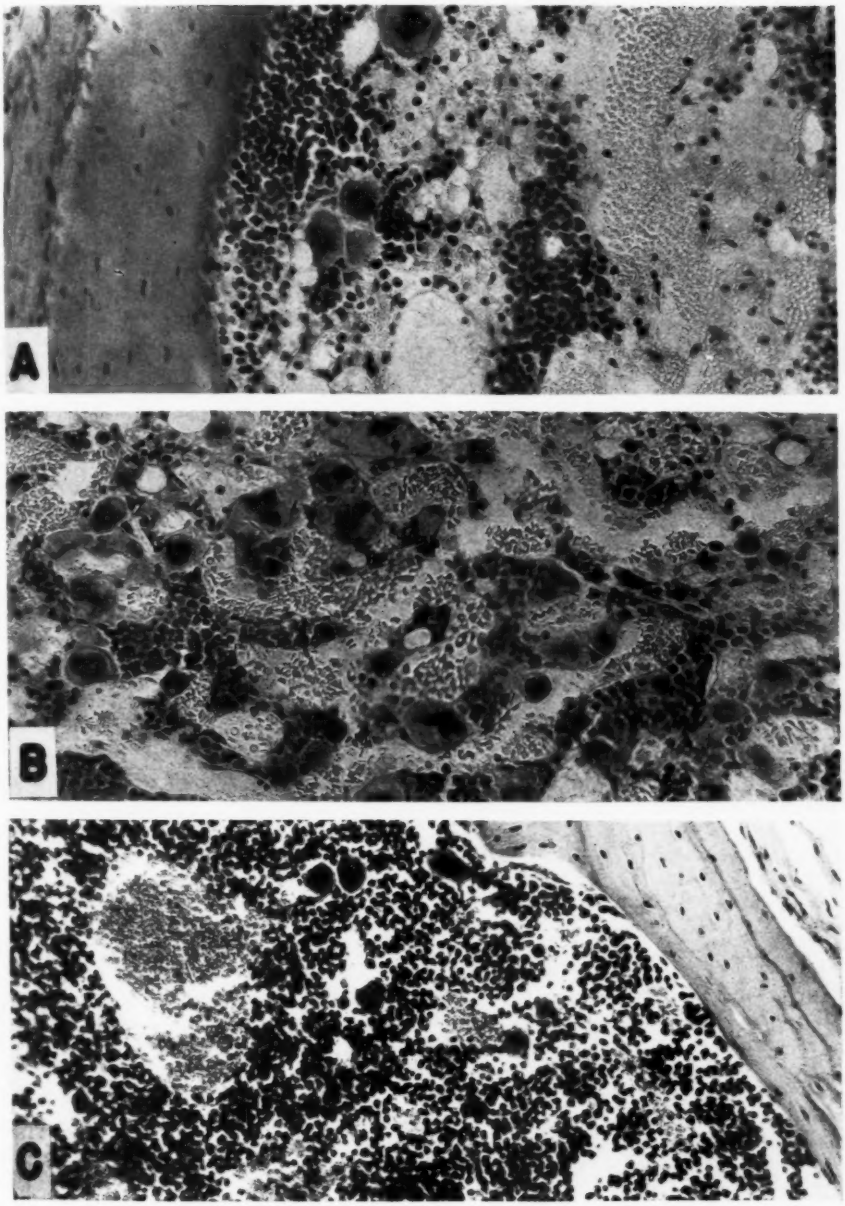

FIGURE 5.—Bone marrow. *A*, One week, *B*, two weeks, and *C*, five weeks after single 440-r dose. Note extreme acellularity in *A*, beginning regeneration in *B*, and nearly normal marrow in *C*. Hematoxylin and eosin stain. × 335.

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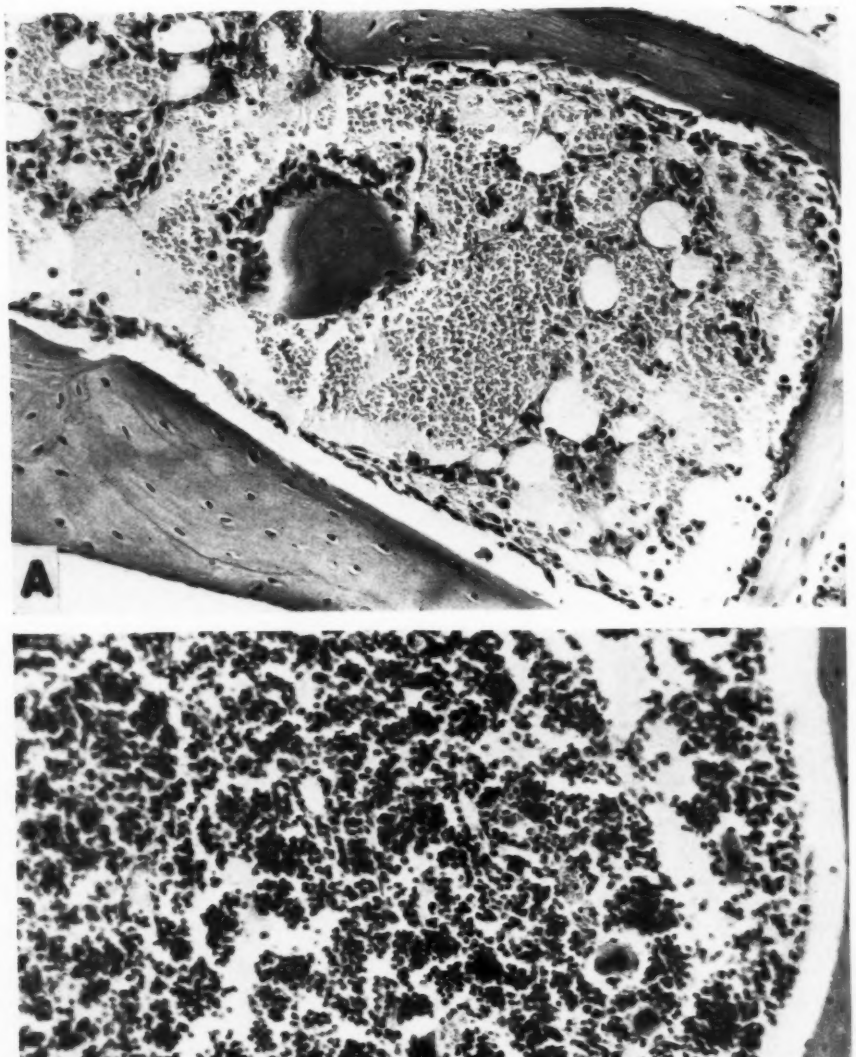

FIGURE 6.—Bone marrow. *A*, Two weeks after second application of 400 r at 10 weeks; *B*, Six weeks after second application of 400 r at 10 weeks. Note remarkable regeneration after extreme marrow destruction. Hematoxylin and eosin stain. × 335.

but the lymphocyte count was rising slowly toward normal. Figure 6, *A*, shows the condition after a second 400-r treatment given 10 weeks after the first. This tissue was fixed at 3 months after the first treatment or 2 weeks after the second. Here the loss of cells was even greater than after the first treatment, but already there was evidence of beginning regeneration. Figure 6, *B*, tissues fixed at 4 months, shows that, despite two drastic tissue insults and severe destruction of the bone marrow, these tissues were nevertheless capable of extensive regeneration. While the degree of regeneration approached the normal range, the character of the regenerative tissue in many cases remained somewhat abnormal. This was made evident by a changed tissue architecture and an increase in number of cells with ring-shaped nuclei (young granulocytes). Considerable hyperplasia was seen in some animals and in still others (seen in other experiments) a frank leukemic invasiveness.

For the animals receiving 12½ and 25 r per treatment, only mild signs of injury could be seen at any time. At 12 weeks, when the animals had accumulated exposures amounting to 200 r or more, the lymph nodes did not show so severe a loss of lymphoid elements, nor did the bone marrow show so many large sinuses. Nevertheless, the lower exposure series developed sustained leukopenia the same as the higher. These differences appear to be associated with the different methods of treatment. When small treatments were given, the fall in lymphocyte level was not carried far below the chosen level of 4,000 r before the treatments were stopped and recovery was permitted. Likewise, only a small amount of recovery was required before treatment was resumed. The diphasic wave of injury and repair at this stage did not have a wide amplitude, and recovery appeared nearly to keep pace with the injury. However, this type of treatment did not prevent the animals from eventually reaching a state of terminal decline, the same as those receiving larger dose treatments.

EXPERIMENTS WITH GUINEA PIGS

Similar experiments with guinea pigs were carried out on a smaller scale. Doses of 25, 50, and 100 r per treatment only were used, and 3,500 lymphocytes per cubic millimeter of whole blood was the level chosen (on the basis of test experiments) as the lower limit of normal. The over-all picture for guinea pigs was the same as that already described for mice, although there were differences in detail. The animals receiving 25 and 50 r per treatment began to show a persisting lower level of lymphocytes after accumulated doses of 250 to 300 r. Those receiving 100-r treatments (two 100-r treatments 3 weeks apart) died during the eighth week after the first treatment.

Normally, the lymphocyte and neutrophil levels in the guinea pig are very nearly the same. In the experiments, the neutrophils and lymphocytes varied simultaneously and in the same direction: and when the animals showed a lymphocyte level persisting in the range around 3,500, the neutrophils dropped into and remained in the range below 3,500. In the animals receiving 25 r per treatment, the neutrophil range was between 1,000 and 3,000, whereas in those receiving 50 r per treatment it was less than 1,000. While the lengthening of the recovery period after a repeated series of treatments tended to vary with the number of series given, it was not so marked as with the mice. It seems clear, nevertheless, from the evidence that somewhat smaller increments of dosage would be necessary to reveal these effects in guinea pigs best. The general histologic changes were much the same as those in mice although the amount of destruction was greater for any given dosage.

DISCUSSION

These experiments add materially to our impression of roentgen injury and the changes which precede irradiation carcinogenesis.

Under normal circumstances, hematopoiesis takes place mainly in the lymph nodes, spleen, and bone marrow,

and throughout life there is a continuous process of blood-cell formation. The erythrocytes, which have to do with oxygen transport and which comprise the main bulk of formed elements of the blood, arise chiefly in the bone marrow. On the other hand, the leukocytes which have to do (to some extent at least) with resistance to toxic agents, arise from both myeloid and lymphoid sources. The life span of erythrocytes in the peripheral blood is understood to be about 30 days or longer whereas that for the leukocytes is 4 to 7 days (*5*, *6*). In connection with the continuous elaboration of blood elements, it is important to remember that not all stem cells give rise only to maturing cells. Some must give rise to other stem forms in order that the parent source can be maintained. The various blast cells appear to be the chief source of most of the blood elements, although back of these are the reticular cells, which form a supporting structure and under certain circumstances give rise to blast forms. The fact that all blood elements arise originally from mesenchymal cells indicates some common relationship between erythrocytes and leukocytes despite their widely differing functions.

In normal life a fairly constant concentration of blood elements must be kept moving through the vessels in order to meet the requirements of the organs and tissues; and under normal circumstances, the rate of production of the various blood elements is very nearly equal to the rate of maturation and loss of these components. There are instances, however, when the demands are excessive (as in case of hemorrhage or disease), and the rate of loss exceeds the rate of production. Under these circumstances, generative tissues are stimulated, and the output is increased in part by means of hyperplasia. When the demands are met, the production rate again subsides. Thus if the stress and strain on hematopoietic organs are not too severe, the various needs of the animal body, so far as blood material is concerned, can be met by means of the reserve capacities of these organs.

Four factors may be mentioned as lending stability to erythrocyte function in higher forms: (1) The relatively long life of erythrocytes; (2) the sinusoidal reservoir of cells in the bone marrow and other parts; (3) the elasticity of the blood vessels; and (4) the ability of the bone marrow to speed up production. It is significant that considerable loss of blood by hemorrhage may occur without particular change in erythrocyte level. This is accomplished in part by a pouring forth of reserve cells, and to some extent by the contraction of the vascular bed so that a uniform blood pressure and cell concentration are maintained despite the lowered total blood volume. By this means, the transportive efficiency of the blood is maintained despite losses. Should temporary injury to the source of new cells, that is, to the hemocytoblasts of the bone marrow occur (e. g., because of generalized toxemia), the transportive efficiency of the blood is maintained again by means of the reservoir of cells and by means of the long life of erythrocytes. The reservoir supplies cells to compensate for loss; and because of the long life of these cells, the reservoir is not used up before the marrow can regenerate and again produce an adequate number of new cells.

Leukocytes, presumably being concerned more with defensive mechanisms than with metabolism, function more in case of emergency than in general maintenance. Injury may occur abruptly and be extensive, causing the demand on leukocytes to suddenly be very much increased. Since such injury must be dealt with in the body by a concentration of cells in the tissue rather than in the circulating blood, it is plain that changes in blood pressure would furnish little aid in meeting the demands. Instead, there must be a migration of cells into the affected region. This is provided for through the maintenance of large reserves of leukocytes which can be drawn upon when emergencies arise. The sudden increase in leukocyte level of several hundred percent in the peripheral blood which accompanies certain acute infections is a familiar picture.

Let us now consider a hypothetical case in which a deleterious agent is applied which causes destruction to blood elements, both in the blood and hematopoietic organs by means of nuclear injury. Let us also examine it in the light of our present impressions. In such a case, the mature erythrocytes would be the only cells unaffected, the others being affected in accord with their relative susceptibility. The lymph nodes, spleen, and bone marrow would show immediate damage, and the blood would show a leukopenia only after the reserve is used up. The erythrocyte level would remain continuously unaffected provided satisfactory regeneration of the bone marrow took place within 3 to 4 weeks.

We, of course, are thinking of an agent which acts on blood elements as does ionizing radiation. The experiments described have demonstrated how remarkably the bone marrow can be destroyed by radiation without producing a significant change in the erythrocyte level. It would appear that when the exposure is acute, recovery is sufficiently rapid to supply an adequate number of cells before the erythrocyte level becomes significantly lessened. Figure 6, *A* and *B*, shows how striking the bone-marrow recovery can be in 4 weeks' time. Because of the unusual regenerative capacity of the bone marrow, as demonstrated here, it would seem that the growth of bone marrow may be sufficient to compensate for a considerable amount of continuously occurring destruction. This probably accounts for the maintenance of fairly normal erythrocyte levels following moderate daily exposures to radiation. That the bone marrow can ultimately be exhausted is adequately demonstrated by the fact that numerous cases of aplastic anemia in overexposed radiation workers have been observed.

With regard to leukocytic elements, the experiments have shown that the application of acute, moderately heavy doses (200 to 400 r) of X-rays produce an extensive breakdown of cellular elements and a fall in leukocyte level within a few hours after treatment which persists for a period of days or weeks. Because of the early appearance of damage in the hematopoietic organs and the quick fall in leukocyte level of the peripheral blood, it would appear that radiation acts destructively on the cells of both the peripheral blood and the hematopoietic organs. The fact that the leukopenias tend to become persistent after repeated treatments suggests that the leukocytic reserve has been exceeded and also that the regenerative capacity of the hematopoietic organs has suffered damage.

In this connection, it is important to compare the reactions of the host organism to irradiation injury and to infection involving leukocyte responses. Suppose first that an infection of virulent type occurs locally at some point on a mammalian organism. The bacteria, although few at the beginning, are multiplying so rapidly that the leukocytes in the region are inadequate to cope with the situation. However, because the infection is localized and the leukocytes are mobile, a great number migrate to the area and the infection is quickly controlled. Suppose secondly that a bacteriemia is present. In this case, bacteria are spread over the entire body, and the leukocytes in each region must deal with the bacteria there present. This condition accounts in part for the seriousness of generalized infection. Radiation in many respects acts like generalized infection. It destroys leukocytes over the entire body regardless of where they may be, and it affects the greatest number of cells in those regions where concentration is greatest. Because of the random action of radiation, it can safely be presumed that relatively the same proportion of leukocytes is injured per unit volume of tissue, irrespective of whether the number is large or small. Thus it becomes understandable why large leukocyte reserves can be exhausted by comparatively small doses of radiation applied to the whole body.

We may now consider what potencies the surviving cells continue to have and what significance can be attached to the persisting lowered lymphocyte

levels which follow a series of X-ray exposures.

First, it appears obvious that the few remaining cells (mainly reticular) give rise to all the blood-cell types (including the neoplastic) which are formed. The tendency toward permanently lowered lymphocyte levels may be explained in one of two ways: Either the capacity of the hematopoietic organs has been reduced, or the mechanism which determines leukocyte levels in the normal body is adjusted downward. From results now available, the first alternative seems the least likely. The curve for guinea pigs (*2, p. 488*) receiving 200 r indicates that an animal showing a depressed leukocyte level can display a marked and transitory leukocyte rise. This fact suggests that some leukocyte reserve is held in abeyance in the usual manner over and above that of the depressed levels.

The persisting leukopenias observed in this series of experiments are not greatly unlike those seen in persons occupationally exposed to radiation (*7*). Just how much lowered levels constitute a health hazard in apparently normal persons has not yet been ascertained.

Fully developed leukemias were not seen in any of the animals studied, although some might have been expected on the basis of previous experience (*8*). The number of animals used, however, was comparatively small, and the duration of the experiment was short for leukemia studies. The histologic material was examined for evidence of neoplasia. Some leukocytic invasiveness showed in a few animals at 4 to 9 months after the beginning of treatment. Most of this was found in animals that received 100 r per treatment and appeared as leukocytic infiltrations in the lungs, slight invasion of the spinal canal and perispinal musculature, and slight invasion of the sternal musculature. In most cases, the predominant cell type was the polymorphonuclear neutrophil which suggested the probability of local infection. Since leukemina was expected in some of these animals, the question is raised whether this condition may not represent an early stage of the disease.

SUMMARY

A series of experiments was carried out to determine the additive effects of different-sized doses of X-rays (0 (controls), 12½, 25, 50, 100, 200, 400 r) applied to the whole bodies of C3H male mice according to a carefully chosen plan.

Four thousand lymphocytes per cubic millimeter of whole blood was set as the lower limit of normal. The treatment for each dosage level was carried forward until the lymphocyte level dropped below this figure, at which time it was discontinued. When 2 consecutive counts above 4,000 were obtained (1 week apart), treatment was again resumed.

The treatment and rest patterns obtained for each dosage group of animals together with the histologic material collected revealed the following: (1) The number of treatments required to lower the lymphocyte level to 4,000 or below became less and period of recovery became longer as the experiment progressed; (2) eventually all of the animals tended to pass into a persisting lymphopenia; (3) in each case, irrespective of the size of the treatment doses, this condition occurred when the accumulated doses were 300 to 500 r.

Histologic examination revealed mild injury in the hematopoietic organs following the repeated small exposures and acute extensive destruction after the large doses. This fact suggests that repair tends to keep pace with destruction in case of the smaller repeated treatments, but that a state of exhaustion is nevertheless eventually reached.

Remarkable regenerative capacities were observed. The bone marrow, for example, returned from an extremely acellular condition to a condition closely resembling normal in 4 weeks.

Similar experiments were performed with guinea pigs using 25-, 50-, and 100-r doses, and 3,500 lymphocytes per

cubic millimeter of whole blood was used as the lower limit of normal. Generally, the same effects were seen as in the case of the mice although the guinea pigs displayed a greater general susceptibility to radiation.

Only mild changes in erythrocyte level were observed in either type of animal at any time.

These findings may be interpreted as follows: (1) The fact that small doses, which singly elicit no detectable change in lymphocyte level, produce a leukopenia after repeated application indicates that subliminal effects are produced and that such effects are additive; (2) this same fact suggests that destruction of lymphocytes occurs with each small dose and that the rate of destruction must go beyond the reserve of lymphocytes and be greater than the rate of production of these cells before lymphopenia can be observed in the peripheral blood; (3) since acellularity in the hematopoietic organs was marked only in those animals that received larger doses, it may be deduced that in the animals receiving smaller doses, the rate of cell production was nearly equal to the rate of destruction; and (4) the persisting lymphopenia may be regarded as the beginning of a state of exhaustion.

The persisting lymphopenia in some respects resembles the slightly lowered leukocyte levels in apparently healthy human beings occupationally exposed to radiation. It is therefore important to watch exposed persons and determine how well they can cope with physiologic stress and strain; and experimentally, it is important to apply load tests to animals with lowered lymphocyte levels in order to ascertain whether they are as able as controls to deal with physiologic emergencies.

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DEGRADATION OF CYSTINE PEPTIDES BY TISSUES. III. ABSENCE OF EXOCYSTINE DESULFURASE AND DEHYDROPEPTIDASE IN TUMORS

By JESSE P. GREENSTEIN, *senior biochemist*, and FLORENCE M. LEUTHARDT, *laboratory technician, National Cancer Institute, National Institute of Health, United States Public Health Service*

In the first two papers of this series (*1*, *2*) it was shown that cystine may be degraded while yet in peptide linkage, yielding by desulfuration the corresponding dehydropeptide which subsequently was hydrolyzed to pyruvic acid and ammonia. The enzyme system responsible was found in liver, kidney, and pancreas; it was absent in spleen, brain, and muscle. The present paper extends this study to various kinds of tumors.

The experimental procedure was identical with that described in the preceding study on normal tissues (*2*). The tumors studied included the following from mice: transplanted hepatomas 587, 98/15, and E, melanomas S91 and Harding-Passey, intestinal adenocarcinoma, pulmonary carcinoma 4461, lymphosarcoma 1885, and the spontaneous mammary tumor in C3H mice. Other tumors investigated were the transplanted Jensen sarcoma in rats, the transplanted fibrosarcoma C2663 in guinea pigs, and the transplanted Brown-Pearce epithelioma in rabbits. The results with these neoplasms, using dichloracetylcystine and chloracetylde-hydroalanine as substrates, respectively, for exocystine desulfurase and dehydropeptidase, were completely negative. These negative results are consistent with those earlier observed by the authors (*3*) when cystine alone was used as substrate with various kinds of mouse tumors.

The exocystine desulfurase-dehydropeptidase system is the only intracellular catalyst so far encountered which, although present in several normal tissues (i. e., liver, kidney, and pancreas), is completely absent in all tumors studied. The fact that this system is apparently absent from so many normal tissues (*2*, *3*) makes it perhaps inadvisable to stress its absence in tumors unduly. Nevertheless, it is possible that tumors as a class may be as well characterized by the lack of certain catalytic systems as by their presence. When liver cells become neoplastic, whether in the rat or in the mouse, most of the chief hepatic functions decrease in activity (*4*), but of these only the systems involving urea synthesis and cystine degradation apparently disappear entirely.

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INDUCTION OF HEPATOMAS IN MICE BY REPEATED ORAL ADMINISTRATION OF CHLOROFORM, WITH OBSERVATIONS ON SEX DIFFERENCES

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Repeated feeding of carbon tetrachloride in olive-oil solution in amounts sufficient to produce liver necrosis will induce hepatomas in mice (*1–4*). Chloroform produces central necrosis of the liver lobule, followed by regeneration of parenchymal cells in a manner similar to that produced with carbon tetrachloride and when given repeatedly will also induce hepatomas. In this report, experimental conditions leading to the induction of hepatomas with chloroform are given in detail while only a brief description of the tumors is given since they appear to be indistinguishable from those induced with carbon tetrachloride. In a previous report (*4*), it was suggested that the cycles of repeated necrosis and repair in the liver might be an important factor in the induction of tumors with carbon tetrachloride. All reports (*1–4*), however, dealt only with the use of necrotizing doses. The use of a graded series of necrotizing and nonnecrotizing doses of chloroform has revealed an apparent necessity for the production of repeated liver necrosis in the induction of these tumors.

EXPERIMENTAL PROCEDURE

Strain A mice from colonies maintained at the National Cancer Institute were used. This strain has an incidence of spontaneous hepatomas of less than 1 percent at 16 months of age (*2*). The mice, which were 3 months of age at the beginning of the experiment, were fed Purina dog chow pellets and tap water in unlimited quantities.

The chloroform was of chemically pure quality and was diluted with U. S. P. olive oil. Five dilutions were used: 32-, 16-, 8-, 4-, and 2-percent solutions. When not in use, the solutions were stored at 6° C. Administration was by stomach tube, and all animals were given repeated doses of 0.005 cc. of solution per gram of body weight per dose, containing respectively, 16×10^{-4}, 8×10^{-4}, 4×10^{-4}, 2×10^{-4}, 1×10^{-4} cc., of chloroform. Control animals received 0.005 cc. of olive oil alone per gram of body weight per dose.

The presence or absence of liver necrosis in relation to dose was determined by microscopic examination of sections of the liver taken 24 hours after a single dose. For this purpose, 1 male and 2 female mice were used for each of the 5 doses. To test for any carcinogenic effect of the chloroform, mice were given 30 doses at intervals of 4 days, this being the condition under which a hepatoma incidence of 100 percent was obtained when carbon tetrachloride was used (*4*). Five male and five female mice were used in each of the 5 experimental and in the control groups. They were examined for the presence of hepatomas 1 month after the last dose, at which time they were 8 months old. Animals of both sexes in each dose group were given 1 additional dose of chloroform solution 24 hours prior to necropsy to determine the relation between dose, the presence or absence of liver and of kidney necrosis, and the occurrence of hepatomas, following repeated administration of the agent.

RESULTS

The occurence of liver and of kidney necrosis in relation to dose and sex is shown in table 1. No sex difference in susceptibility to liver necrosis was observed. In both males and females extensive necrosis of the liver was produced with the three largest doses, whereas no necrosis was observed following administration of the two smallest doses. A striking sex difference was observed, however, in the occurrence of renal necrosis, which was present in all males in each of the five dose groups and

absent in all females so treated. Necrosis of the liver and the kidney is described in the section on pathology.

Table 1 also shows the deaths and tumor incidence in relation to dose in surviving animals in the experiment to test for carcinogenicity of chloroform. Death came 24 to 48 hours after the first or second dose. No animals survived repeated administration of the largest dose, and no males survived the 3 largest doses. All females that survived repeated administration of the 3 largest doses had hepatomas, but no hepatomas

TABLE 1.—*Liver and kidney necrosis and hepatomas in strain A mice following repeated oral administration of chloroform in olive oil*

[Numerators = positive occurrences; denominators = animals observed]

Observation	Sex	Dose [1]					Control
		16×10^{-4}	8×10^{-4}	4×10^{-4}	2×10^{-4}	1×10^{-4}	
Liver necrosis	F	+	+	+	0	0	0
	M	+	+	+	0	0	0
Kidney necrosis	F	0	0	0	0	0	0
	M	+	+	+	+	+	0
Deaths	F	5/5	1/5	2/5	0/5	0/5	0/5
	M	5/5	5/5	5/5	2/5	0/5	0/5
Hepatomas in surviving animals receiving 30 doses.	F	....	4/4	3/3	0/5	0/5	0/5
	M	....	....	....	0/3	0/5	0/5

[1] Cubic centimeters chloroform per gram body weight per dose (30 doses).

were observed after the 2 smallest doses had been given repeatedly. The sharp end point for carcinogenicity thus coincided with the end point for liver necrosis in relation to dose. No males that received doses large enough to produce liver necrosis remained alive after repeated administration of the agent, and none was observed to have hepatomas. No anesthetic properties of chloroform were apparent in any mice after oral administration, even though the largest dose was fatal to all 10 mice to which it was given.

PATHOLOGY

The animals were killed by cervical dislocation, and the tissues were fixed in Zenker-formol, embedded in paraffin, and stained routinely with hematoxylin and eosin. Some sections were also stained with Laidlaw's stain for reticulum. Sections were prepared of kidneys and livers of all animals and of other organs and tissues of several animals of each dose group.

Twenty-four hours after a single dose of chloroform, there was extensive necrosis of liver cells around central veins. The line of demarcation between necrotic and apparently normal liver cells was sharp. The two largest doses produced extensive central necrosis of all lobules of each lobe of the liver while the next smaller dose (4×10^{-4} cc.) produced central necrosis of lobules of only some lobes. In the latter case, however, there was either necrosis of 15 percent or more of liver cells in a given lobule or no discernible necrosis.

The renal necrosis that was observed in male but not in female animals of each dose group involved portions of both proximal and distal convoluted tubules. The nuclei of the epithelial cells were often absent or fragmented, and the cytoplasm was coarsely granular and deeply eosinophilic. The glomeruli and collecting tubules appeared normal. A sex difference in the structure of Bowman's capsule in mice, described by Crabtree (*6, 7*), was confirmed in the following observations. The kidneys of 15 female and 14 male mice 24 hours after administration of chloroform were available for study. In the female mice, Bowman's capsule of most of the glomeruli was lined completely with flat epithelium, while in the males Bowman's capsule of most of the glomeruli was lined partially or completely with cuboidal epithelium indistinguishable from the epithelium of the associated proximal convoluted tubule. The cuboidal cells lining Bowman's capsule and the cells lining the upper portions of the proximal convoluted tubules were never necrotic, even when necrosis was present in lower portions of these tubules. The necrosis of the kidneys in male mice was always widespread in contrast with complete absence of necrosis in kidneys of female mice. However, it was less extensive

in male mice that had received smaller doses of chloroform.

Animals that received 30 doses of chloroform at intervals of 4 days and were killed 1 month after the last dose had moderately cirrhotic livers. Grossly, the surfaces of the livers were slightly nodular although the general contours of the lobes were preserved. Microscopically, there was moderate portal fibrosis, which was found, with the use of special stains, to consist of large amounts of compact reticulum. There was also slight increase of reticulum throughout the lobules, and the interportal margins of the lobules were outlined by more dense deposition of fine reticulum fibers. Small calcium deposits were observed in some of the areas of fibrosis. The livers of animals receiving doses of chloroform too small to produce necrosis (2×10^{-4} and 1×10^{-4} cc. per gram body weight) appeared normal in sections stained with hematoxylin and eosin and with Laidlaw's stain for reticulum.

The hepatomas resembled those induced with carbon tetrachloride (*2*), from which they could not be distinguished morphologically in this preliminary study. They were composed of liverlike cells arranged in cords one or two cells in thickness, between which were endothelial-lined blood sinuses. The cords of cells, however, did not radiate from central blood vessels but formed disorderly anastamosing columns. Although there was considerable variation in size of the hepatoma cells, their average size was noticeably greater than that of the normal liver cells. Mitoses were not very numerous. Dalton observed that the mitochondrial pattern of these cells was similar to that he previously reported (*5*) for other induced hepatomas and different from that of spontaneous hepatomas in the mouse. The tumors were circumscribed by compressed liver tissue, but they were not encapsulated by fibrous tissue and never appeared invasive. No metastases were observed. In all instances in which an additional dose was given 24 hours prior to necropsy, there was focal necrosis of liver cells of all lobules (except when the dose was 4×10^{-4} cc. per gram body weight, when lobules of only some lobes were affected) and no necrosis of hepatoma cells.

DISCUSSION

It was previously pointed out (*4*) that carbon tetrachloride produces necrosis of liver cells in the center of the lobules, followed by rapid multiplication of liver cells that escape necrosis. It was suggested that such a cycle when repeated many times might permit a progressive selection of the liver cells that have the greatest potentiality for division and for resistance to the lethal action of the agent. The question was raised whether such a cyclic process plays any significant role in the genesis of these tumors. The present limited data on hepatomas induced with chloroform are of interest relative to this question, for it was found that hepatomas and cirrhosis of the liver were induced only if individual doses were large enough to produce necrosis of the liver, and that the hepatomas were resistant to the necrotizing action of the agent. These data do not, however, rule out the possibility that hepatomas may be produced with nonnecrotizing doses of chloroform, given under different conditions with regard to number and spacing of doses, for these were found to be important factors in the incidence of hepatomas produced with carbon tetrachloride when the total quantity administered was constant (*4*). In this connection, the observations of Bollman and Mann (*8*) on the induction of cirrhosis of the liver in dogs are of interest:

> The chronic lesions of the liver we have been able to produce depend on the acute lesions repeatedly produced. Changes are not observed with frequent doses which are so small that acute necrosis is not produced.

A similar conclusion may be drawn from the data of Cameron and Karunaratne (*9*) in the induction of cirrhosis of the liver in rats with carbon tetrachloride. These considerations recall a statement made by Edwards (*1*) in his first report on the induction of carbon tetrachloride hepatomas:

Whether carbon tetrachloride is the active agent in inducing hepatomas in mice or whether these tumors are merely the result of hepatic damage caused by carbon tetrachloride awaits further study.

Following oral administration of 4 x 10^{-4} cc. of chloroform per gram body weight in olive-oil solution, central necrosis of liver lobules was observed in some lobes of the organ only. The distribution of these necrotic lobules in relation to the various lobes was not determined in this experiment. From the observations of Copher and Dick (*10*) on the "stream line" phenomena in the portal vein of dogs, a selective distribution of portal blood in the liver, from different parts of the gastrointestinal tract, seems a more likely explanation of this finding than that of a difference in susceptibility of liver cells in different lobes. Following oral administration of carbon tetrachloride in olive oil, however, minimum necrotizing doses produced necrosis in all lobules of the liver,[1] suggesting that absorption of these two chemicals occurs in different portions of the gastrointestinal tract.

The observation that no anesthetic properties of chloroform were apparent prior to the death of animals 24 to 48 hours after oral administration of the largest dose suggests that the liver filtered out most of the chemical present in the veins draining the gastrointestinal tract. In these animals there was necrosis of approximately 50 percent of the liver cells. In order to produce liver necrosis with this agent given by the respiratory route, it is necessary to produce prolonged deep anesthesia. If the renal necrosis in male mice was caused primarily by chloroform, this chemical must have been active in extremely small quantities. An alternative explanation of the renal necrosis in male but not in female mice might be found in a sex difference in toxic products formed by the reaction of other tissues to chloroform. It should be recalled, however, that renal necrosis was observed in male mice in the absence of liver necrosis following doses of 2 x 10^{-4} and 1 x 10^{-4} cc., respectively, of chloroform per gram of body weight.

The finding of a sex difference in susceptibility to renal necrosis is of interest in view of differences in the structure of Bowman's capsule and in proteinuria in normal mice. In 1939 Selye (*11*) recorded that following injection of testosterone propionate into female mice there occurred hypertrophy of the epithelium of the proximal and distal convoluted tubules and of the epithelium lining Bowman's capsule. Commenting on this report in 1940, Crabtree (*6*) pointed out that a sex difference in the structure of Bowman's capsule is seen in normal mice. She observed that the epithelium lining most of these structures in adult female mice is flat whereas in adult male mice it is partly or entirely cuboidal. Subsequently Wicks (*12*) reported the finding of extensive proteinuria in males and a trace to none in females of nine different strains of mice, including strain A. In the present experiments, a sex difference in the susceptibility to renal necrosis and in the morphology of the epithelium lining Bowman's capsule was noted. Further experiments would be necessary to determine in what respect these two findings are related.

SUMMARY

Chloroform was found to induce hepatomas and cirrhosis of the liver in strain A mice.

These lesions occurred after repeated oral administration only if individual doses were large enough to produce liver necrosis. The tumors could not be distinguished histologically from those induced with carbon tetrachloride. They were resistant to the necrotizing action of chloroform.

Renal necrosis occurred in male but not in female mice, while no sex difference in susceptibility to liver necrosis was observed. Doses sufficient to produce liver necrosis caused lethal kidney

[1] Unpublished data.

necrosis in male mice. Therefore, hepatomas were observed only in female mice.

Doses of chloroform that were lethal to both sexes resulted in death of the animals 24 to 48 hours after oral administration, without the appearance of the anesthetic qualities that characterize this agent when it is given by the respiratory route.

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MULTIPLE TRAY FOR EMBEDDING TISSUES IN PARAFFIN

By ALBERT M. KESSEL, *senior medical technician, National Cancer Institute, National Institute of Health, United States Public Health Service*

The embedding of tissues or other objects in paraffin is one of the most important steps in the process of preparing paraffin sections. Difficulties frequently occurring during this process are (1) local or general cooling and solidifying of the paraffin during embedding, (2) local or general overheating of the paraffin, and (3) where paper boxes are used as molds, the loss of time in preparing them.

The device described herein has been used in this laboratory for some time and has substantially reduced the difficulties enumerated, while it has also increased the ease and accuracy of orientation of the objects within the block. In conjunction with the multiple tissue carrier,[1] it has resulted in a considerable saving of time in the embedding of large numbers of routine pathologic objects.

The assembled tray (fig. 1) consists of 5 units, of 10 compartments each, 1 compartment being the size of 1 block. The 5 units are separated by 4 strips. The assembled units rest on a plate with engraved numbers to identify the individual compartments and the cast-paraffin blocks. The plate, compartments, and strips fit into a pan which is placed on a rectangular, electrically heated plate [2] like those usually used to dry slides. This latter plate is the same size as the pan and is held at constant temperature.

The complete device consists of 50 compartments, each of which can be divided into 2 or 3 blocks, depending on the size of tissues. It can be made of any one metal or combination of metals suitable for paraffin embedding, and the dimensions may be varied according to the needs of the laboratory and equipment already available. For the units and the dividing strips, half-hard brass strips 1 by $\frac{1}{8}$ inch thick were used. Details of the construction are as follows:

The pan (fig. 2, *A*), which is tapered on all four sides, is made of galvanized sheet metal $\frac{1}{16}$ inch thick. It is $22\frac{3}{4}$ inches long at the bottom, and 23 at the top; $5\frac{1}{4}$ inches wide at the bottom, and $5\frac{1}{2}$ at the top; and $1\frac{1}{4}$ inch high.

The identification plate (fig. 2, *B*) is made of brass and measures $22\frac{3}{4}$ by $5\frac{1}{4}$ by $\frac{1}{8}$ inch thick and fits the bottom of the pan. The numbers (1 to 50) are engraved in reverse from left to right and are equally spaced in the middle of each compartment. They are $\frac{3}{8}$ by $\frac{3}{8}$ inch and about $\frac{1}{16}$ inch deep. When the block is removed from the plate, it can be identified by the number, which stands above the paraffin surface. If the plate cannot be constructed, identification may be made possible by placing a paper label in each compartment.

[1] KESSEL, A. M.: Multiple tissue carrier for histologic techniques. J. Nat. Cancer Inst., 4: 361–362 (1944).

[2] Type 26, sold by Arthur Whiteman Corp., New York.

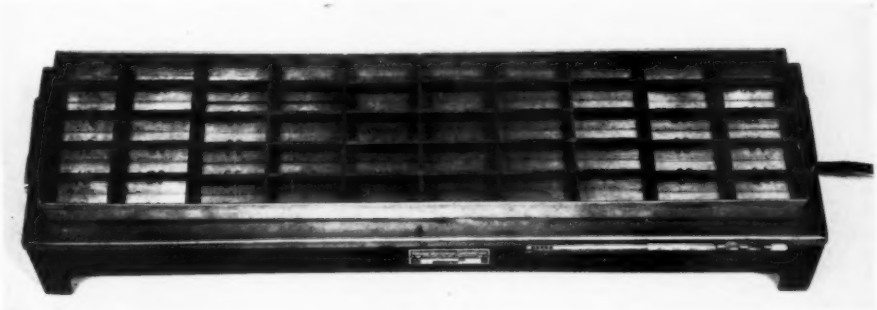

FIGURE 1.—Assembled multiple paraffin-embedding tray, with electrically heated plate.

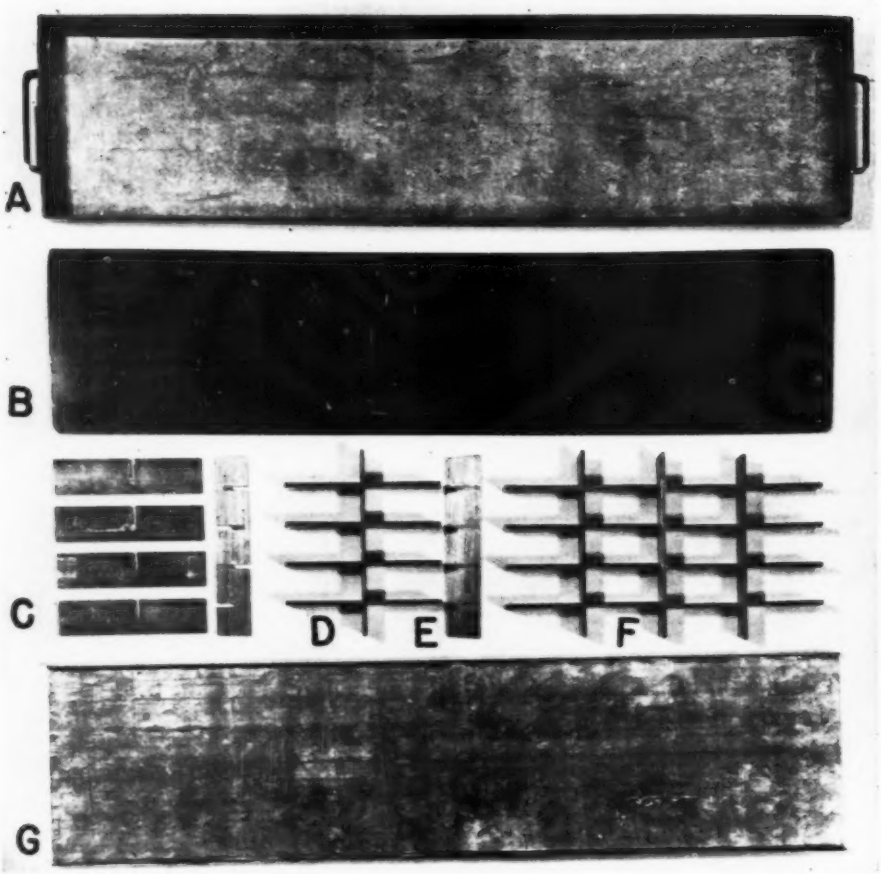

FIGURE 2.—*A*, Pan; *B*, Identification plate; *C*, Parts of one unit; *D*, Unit assembled; *E*, Strip; *F*, Two units assembled, with strip in center; *G*, Cover for pan.

Each compartment (fig. 2, *C*) is made of five rectangular brass strips. The longest strip, which is 5¼ inches at the bottom, 5½ at the top, 1 inch high, and ⅛ inch thick, has four equally spaced slots, ⅛ inch wide, ⅛ thick, and ½ inch deep; two of them are at the bottom and two at the top. The four cross strips each measure 4½ inches long, 1 high, and ⅛ inch thick, with one slot in the middle of the same dimensions as the slots in the long strip. When assembled, the four cross strips fit slot into slot in the long strip so that they form one dividing unit (fig. 2, *D*). All edges toward the side of the pan are tapered to ¼ inch.

Each of the four strips (fig. 2, *E*) used to separate the units (fig. 2, *F*) is 5¼ inches long at the bottom, 5½ at the top, 1 inch high, and ⅛ inch thick.

The cover (fig. 2, *G*) is a straight piece of galvanized sheet metal which fits over the pan and is bent on two sides to prevent it from sliding off the pan.

After the tray is assembled for use, the inner surface of the pan and compartments should be lightly coated with vaseline to prevent adherence of the paraffin to the metal, and the tray should be placed on the electric heated plate. This is best done 1 hour before starting embedding. Then the paraffin is poured into the pan; the tissues are placed in the bottom of the tray, warmed forceps being used, and are orientated so that they are conveniently situated for trimming the paraffin block prior to sectioning. Each tissue should be so placed that the part at which sectioning is to begin lies face downward. After the tray is filled, it is best to cool it as much as possible, either in ice water or in a refrigerator. When the tray is cool, it is turned upside down and tapped lightly on all sides; the plate with all the compartments will fall out. The plate should be removed first, then the paraffin blocks (fig. 3). The tray can be cleaned with xylene and is ready for use again.

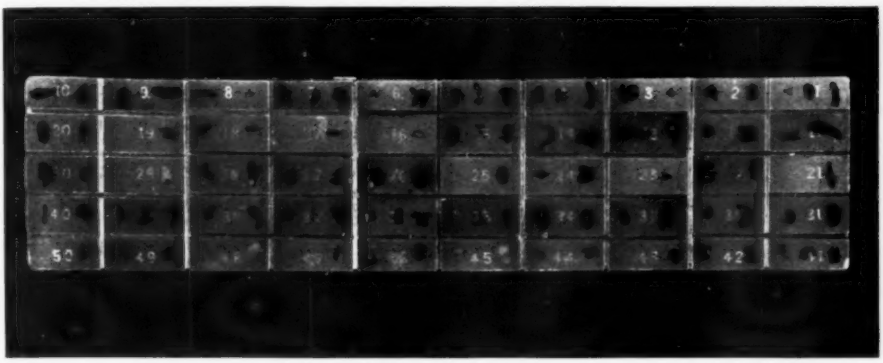

FIGURE 3.—Paraffin blocks, with tissues embedded, removed from the tray.

SOURCE OF TUMOR PROTEINS. I. EFFECT OF A LOW-NITROGEN DIET ON THE ESTABLISHMENT AND GROWTH OF A TRANSPLANTED TUMOR [1]

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A tumor is a growing tissue and like developing organisms deposits protein in the form of protoplasm and other constituents in each cell formed. The origin of such newly deposited nitrogenous material in growing animals is dietary protein. However, such need not be the case in the growth of a tumor, since, theoretically at least, the normal tissues of the host are also available to it as a supply of protein.

Without the use of isotopes, the ideal way to study the source of the newly deposited protein in neoplasms would be to transplant a tumor into a starving mouse and determine the amount of protein in the tumor, provided any neoplastic growth would result under these conditions. The survival period of such a mouse would be so short as to allow, at best, little more than the establishment of the transplant. However, after glycogen and fat reserves are exhausted, a starving animal uses tissue protein for essential energy expenditure as well as for protein catabolism. Accordingly, if body protein could be conserved for endogenous processes only and the energy needs of the animal met by a protein-free supply of foodstuffs, the survival period of the mouse would be sufficiently extended to permit a study of whether or not a transplanted tumor would become established and grow without a source of dietary protein. If such tumors did take, then their growth rates could be compared with those of similar tumors in mice fed an adequate diet. The purpose of the work reported in this paper was to make such a study.

EXPERIMENTAL PROCEDURE

Young albino mice of strain-A-backcross derivation, both sexes, were used for the study. One group, selected at random, was fed an adequate diet, and the other similar group, a low-nitrogen diet. The diets are given in table 1. After a week of adaptation to the diet, one uniform-sized (2.5 cubic millimeter) piece of tumor 15091A [4] was implanted subcutaneously in the back of each mouse. At intervals, the size of the tumor in three dimensions was measured with the aid of calipers. Since such measurements were subject to considerable inaccuracy because of the covering of skin and to distortion in attempting to measure thickness, each tumor was dissected free at necropsy and measured carefully. These measurements agreed within 10 percent with those made immediately before death.

TABLE 1.—*Composition of diets*

Constituents	Adequate diet	Low nitrogen diet
	Percent	*Percent*
Casein [1]	18	0
Cornstarch	41	59
Inorganic salts [2]	6	6
Cod-liver oil	3	3
Crisco	22	22
Ryzamin B No. 1 [3]	5	5
Liver extract [4]	5	5
	Milligrams	*Milligrams*
Nitrogen per gram of food	30.7	5.6

[1] Casein No. 453, The Casein Co. of America, Inc.
[2] Osborne-Mendel salt mixture (1).
[3] Burroughs Wellcome & Co., Inc.
[4] Extract No. 343, Eli Lilly & Co.

The mice were killed by decapitation; and the blood was collected with potassium oxalate for the determination of amino acid nitrogen, which was done by the method of Folin (*3*) using an Evelyn photoelectric colorimeter with a green filter of 520 $\mu\mu$. It was necessary to pool the blood from several mice to

[1] This investigation, in part, was from the Laboratory of Physiological Chemistry and the Department of Pathology, Yale University School of Medicine, and was aided by a grant from the Hubbard-McCormick Clinical Cancer Research Fund.

[2] Now at the Department of Pharmacology, Medical College of the State of South Carolina, Charleston, S. C.

[3] Sincere appreciation is expressed to Dr. C. N. H. Long of the Department of Physiological Chemistry, Yale University School of Medicine, for his advice and encouragement throughout the course of this investigation.

[4] A mammary adenocarcinoma which arose spontaneously in a strain A mouse and was carried in albino mice of strain-A-backcross derivation.

have an adequate amount for a determination. The results are given in table 2.

RESULTS AND DISCUSSION

The liver extract was, of course, composed of protein split-products and could, insofar as it contained sufficient of the essential amino acids, spare body protein. To test this point, weanling strain dba mice were found to maintain their weight rather well on a diet consisting of the following: liver extract, 20 percent; Crisco, 22 percent; cod-liver oil, 3 percent; inorganic salts, 6 percent; Ryzamin B, 5 percent; and cornstarch, 44 percent. Excellent growth resulted when the nine essential amino acids (no arginine) in the quantities used by Rose and Rice (*2*) replaced an equivalent amount of starch. By the omission of one essential amino acid at a time, the resultant growth curves indicated that at a 20-percent level the liver extract probably contained all the leucine, methionine, phenylalanine, valine, and possibly all the tryptophane needed for growth, no lysine or histidine, and a suboptimal amount of isoleucine and threonine. Thus, at a 5-percent level, as the liver extract was used in the low-nitrogen diet, the mice into which transplants of tumors were made were definitely restricted in at least four and possibly more essential amino acids.

As shown in table 2, there was no apparent difference in the number of successful tumor transplants between the two groups of mice or in the time of appearance of the earliest palpable lump. These are really astonishing ob-

TABLE 2.—*Effect of protein intake on the growth of a transplanted tumor in mice.*

Sex of mice	Mice on low nitrogen diet							Mice on adequate diet						
	Volume of tumor at—				Body weight		Amino Acid nitrogen of blood (mg./100 cc.)	Volume of tumor at—				Body weight		Amino Acid nitrogen of blood (mg./100 cc.)
	7 days	10 days	14 days	21 days	Start [1]	End		7 days	10 days	14 days	21 days	Start [1]	End	
	Mm.[3]	*Mm.*[3]	*Mm.*[3]	*Mm.*[3]	*Grams*	*Grams*		*Mm.*[3]	*Mm.*[3]	*Mm.*[3]	*Mm.*[3]	*Grams*	*Grams*	
F	------	L [2]	896	1,540	16.4	10.9		------	L	600	1,800	14.3	19.5	
F	------	------	------	------	16.2	11.0		------	L	874	11,520	14.2	18.5	7.75
F	------	------	L	1,430	17.4	12.4		------	L	1,620	5,594	14.8	17.4	
F	L	320	818	1,078	16.8	12.3	6.38	------	L	L	[3]	15.6	19.5	
F	L	126	1,120	2,800	16.4	11.9		L	256	2,970	11,850	15.5	20.2	8.30
F	L	280	672	1,297	16.4	12.9		L	72	734	3,890	15.2	19.4	
F	L	210	2,700	4,930	16.1	12.7		------	240	3,025	7,560	14.6	18.9	
F	------	L	84	616	17.2	12.4		------	L	2,700	3,300	15.3	[4]	
F	------	45	546	3,430	15.8	13.4		------	L	1,400	5,000	14.8	19.7	7.91
F	L	120	144	810	16.0	12.1		------	------	700	4,720	14.0	15.5	
F	L	L	198	2,420	15.2	13.7		L	60	2,520	5,470	14.5	19.1	
F	L	L	288	1,458	16.1	13.7	7.36	------	360	1,960	6,260	15.2	18.2	
F	------	L	64	525	15.8	11.7		L	1,872	5,120	13,120	15.0	19.6	7.82
F	L	L	36	467	15.4	10.8		------	768	1,410	8,780	15.0	19.4	
F	L	L	1,200	1,952	15.7	13.4		L	96	700	2,160	12.2	16.2	
F	L	480	960	960	15.5	12.4		L	128	1,680	5,540	15.1	19.7	8.75
M	L	264	1,600	2,760	23.4	16.8		L	105	588	1,705	24.7	26.2	
M	------	L	140	864	25.2	17.5		L	96	1,540	3,700	24.5	23.5	8.50
M	L	618	2,200	3,480	24.6	16.4	7.20	------	560	3,890	11,250	26.2	25.5	
M	------	L	480	4,350	23.4	17.2		------	------	84	8,100	25.1	26.8	7.96
M	------	------	L	192	21.6	16.4		------	120	3,500	9,860	22.3	22.8	
M	------	L	810	3,770	22.4	17.6		L	1,440	3,600	9,900	25.3	22.5	
M	------	280	350	2,530	22.4	15.7		------	L	720	7,140	22.1	24.0	8.24
M	------	252	943	2,200	24.4	15.8	6.84	------	1,387	4,200	9,000	21.4	22.7	
M	L	192	2,110	6,300	24.2	19.8		L	3,890	4,175	8,570	20.5	21.6	
M	L	60	2,500	4,800	22.7	17.2		------	L	1,080	6,330	20.2	23.5	
M	------	180	1,232	5,520	24.5	17.8		------	------	L	123	21.2	25.1	8.24
M	------	420	2,160	2,640	20.2	[4]		------	L	L	112	22.1	23.9	
M	------	------	------	------	19.4	14.3	7.00	------	------	------	[3]	21.2	[4]	
M	L	648	1,850	4,500	18.7	14.8		------	L	1,760	4,800	22.0	22.6	8.04
M	L	L	27	400	18.5	13.1		------	------	------	------	21.2	23.4	

[1] When tumor was transplanted, after 1 week on the diet.
[2] L indicates very small tumor, 27 mm.[3] or less.
[3] Tumor largely in peritoneal cavity.
[4] Nearly dead so not weighed. Not included in blood amino acid nitrogen determination.

servations in view of the differences in diet. The low-nitrogen-diet group received in their food at best only the meager amount of incomplete protein fragments contained in one of the vitamin supplements. They were in negative nitrogen balance at all times (*4*) and lost a quarter of their body weight during the 3 weeks. Nevertheless, this lack of dietary protein resulted in no apparent interference in the establishment of the tumor transplant. There was, however, a statistically significant alteration in the growth rate of the tumors. To establish this point, the procedure discussed by Blum (*5*) was used for the determination of a constant, K, to express the rate of growth for each tumor. The mean values of K for the tumors of animals in each group were found and by use of the t statistic (*6*) were shown to differ significantly ($P < 0.01$). The mean K value for 7 days of the growth rate of tumors among the mice fed a normal diet was 1.336, while that of tumors in mice fed the low-nitrogen diet was 0.976. This indicates that the transplanted tumors in the mice whose diet contained no protein, grew at nearly 74 percent of the rate of tumors in normally fed mice. The final sizes of the three largest tumors of the nitrogen-restricted group were 35x15x12, 23x20x12, and 28x16x11 mm. These were fairly large tumors and represented the deposition of at least a gram of new protein. The food, at an average intake of 3 gm. per day, supplied about double the amount of nitrogen that this new protein represents. However, the protein split-products of the food were devoid of two and deficient in at least two other essential amino acids. During this 3-week period of tumor growth, the negative nitrogen balance mentioned indicated that the endogenous need for protein nitrogen of the mouse exceeded that supplied by the diet. Thus, the conclusion that a tumor is able to draw upon the tissues of the host, if necessary, to supply its metabolic needs is strongly indicated. Furthermore, this ability to make demands upon the host was modified surprisingly little even by the extreme conditions imposed in this experiment. In the face of protein starvation, while the growth rate was somewhat slowed, very little effect upon the establishment of a tumor transplant was evident.

SUMMARY AND CONCLUSIONS

Tumor transplants in mice fed a diet of which the protein content was limited to the small amounts contained in the vitamin supplement grew at 74 percent of the rate of similar transplants in mice fed a normal diet. Protein deprivation exerted no apparent effect upon the establishment of the transplant. This fact strongly suggests that a tumor is able to use the tissues of its host to supply its metabolic needs when necessary.

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SOURCE OF TUMOR PROTEINS. II. NITROGEN-BALANCE STUDIES OF TUMOR-BEARING MICE FED A LOW-NITROGEN DIET [1]

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In the preceding paper [3] it was shown that tumor transplants became established and grew in mice whose diet contained no protein as such. It seemed probable that these tumors grew, partly at least, at the expense of the tissues of their host. However, to establish this fact definitely, a more detailed study seemed desirable.

The plan of the experiment was to set up a balance sheet for each mouse to account for all changes or redistributions of nitrogen between the food, excreta, tissues of the host, and tumor tissue during the growth of the tumor. If the amount of nitrogen in the mouse itself at the start of the experiment and that in the food consumed during the experiment were determined, the sources of nitrogen for the formation of new cells in the growing tumor would be known. By analyzing in addition all excreta collected during the experimental period, the dissected tumor, and the remainder of the mouse at the finish, shifts occurring to account for the nitrogen found in the tumor might be evident. Particularly from a study of such data from mice fed a diet without protein would it be possible to determine whether the tissues of the host had supplied any of the nitrogen used in the tumor.

EXPERIMENTAL PROCEDURE

Young adult albino mice of strain-A-backcross derivation of both sexes were selected at random. As controls some were killed by cerebral dislocation; the entire mouse was digested with sulfuric acid and selenium as a catalyst, and aliquots of the digest were analyzed for nitrogen. Other similar mice, in groups of six at a time, received subcutaneous transplants of tumor 15091 A.[4] They were immediately placed in the metabolism cages (fig. 1) and fed the

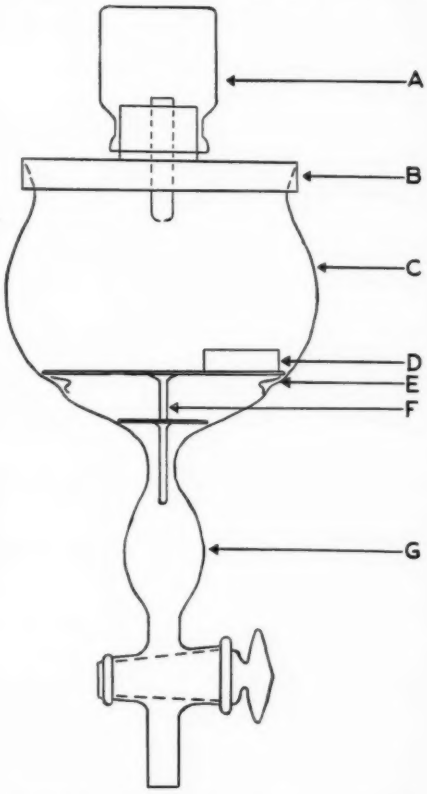

FIGURE 1.—Metabolism cage for a mouse. *A*, Water bottle; *B*, Lid, a copper band with center of 4-to-the-inch hardware cloth; *C*, Adapted from a 500-cc. Florence flask; *D*, Circular metal collar to hold food cup in place; *E*, Indentations in the glass wall to help support platform; *F*, Two parallel wire-mesh platforms soldered to a brass center wire. Upper platform 4-to-the-inch mesh. Lower platform 16-to-the-inch screen wire; *G*, Bulb, capacity about 10 cc.

[1] This investigation was carried out at the Laboratory of Physiological Chemistry, Yale University School of Medicine, and was aided by a grant from the Hubbard-McCormick Clinical Cancer Research Fund.

[2] Sincere appreciation is expressed to Dr. C. N. H. Long, of the Department of Physiological Chemistry, Yale University School of Medicine, who suggested this problem, for his advice and encouragement.

[3] WHITE, F. R., and BELKIN, M.: Source of tumor proteins. I. Effect of a low-nitrogen diet on the establishment and growth of a transplanted tumor. J. Nat. Cancer Inst., 5: 261–263 (1945).

[4] A mammary adenocarcinoma which arose spontaneously in a strain A mouse and was carried in albino mice of strain-A-backcross derivation.

experimental diets described in the preceding paper. In each group of six mice, three received the 18-percent casein and three the protein-free diet. The mice were weighed, the food intake was determined, and the urine was collected and analyzed for total nitrogen each day. Except in the case of one group of six mice, feces were collected daily and saved for analysis at the conclusion of the experiment. When the continued life of the mouse became doubtful, it was killed by cerebral dislocation, and the tumor was dissected free and analyzed for nitrogen. The remainder of the mouse was analyzed separately for total nitrogen. Each batch of food was also analyzed for nitrogen and found to vary between 4.8 and 5.9 mg. per gram of low-nitrogen diet and between 29.0 and 31.7 mg. per gram of 18-percent-casein diet. All nitrogen determinations were done by micro-Kjeldahl distillation and titration on aliquots of urine and food, or aliquots of digests of stool, mouse carcass, and tumor. Nitrogen determinations on known compounds were made from time to time as checks.

RESULTS AND DISCUSSION

Table 1 gives the results of the analyses of whole, normal, young mice taken from stock. The variation in amount of nitrogen per unit of body weight was large and made the assumption of a value for the nitrogen content of a mouse at the start of the experiment on the basis of body weight, of doubtful accuracy. If a 20-gm. mouse had been chosen for a balance study, the nitrogen content of its tissues and intestinal contents (which later would be either tissue, urinary, or fecal nitrogen, and determined as such in the course of the study) would be 548 mg., if the average value given in the table was used, but might actually vary 100 mg. above or below this figure, or more than the total amount of nitrogen found in most of the tumors. Hence this part of the proposed balance study was necessarily omitted.

TABLE 1.—*The nitrogen content of whole albino mice of strain-A-backcross derivation (ABC)*

Sex of mice	Body weight	Total nitrogen	Nitrogen per gram body weight
	Grams	*Milligrams*	*Milligrams*
Female	23.8	727.8	30.6
Do.	24.2	775.0	32.0
Do.	27.7	850.5	30.7
Do.	27.6	803.0	29.1
Do.	23.5	621.5	26.4
Do.	27.9	725.5	26.0
Do.	26.9	644.5	23.9
Do.	27.9	627.5	22.5
Do.	25.7	678.5	26.4
Do.	30.8	726.5	23.6
Do.	30.0	695.2	23.2
Do.	26.9	727.0	27.0
Do.	18.8	518.0	27.5
Do.	17.8	498.5	28.0
Male	26.9	833.3	31.0
Do.	25.2	786.5	31.2
Do.	25.8	790.0	30.6
Do.	22.1	642.5	29.1
Do.	21.9	618.5	28.3
Do.	19.2	517.0	26.9
Do.	23.9	674.5	28.2
Average			27.4

The results of the remainder of the analyses are given in table 2. The two mice on the low-nitrogen diet without tumor transplants excreted an average of 16.4 and 17.2 mg. of nitrogen a day, respectively. By the third day of starvation following normal feeding, the urinary nitrogen of mouse No. 3 was 17.1 mg. while on the sixth day the excretion of mouse No. 4 had fallen to 22.0 mg. The fact that the excretion of nitrogen was no greater when the low-nitrogen food was fed than when the mouse was starved indicated that the nitrogen of the protein split-products present in the liver extract of the diet were able to spare tissue protein. This seemed to be true, even though it was previously shown that at the level used, the liver extract was inadequate in at least four essential amino acids. However, in spite of the tissue-sparing action of the low-nitrogen diet, none of the mice eating it, with or without tumors, were ever in other than negative nitrogen balance. The extent to which over-all loss exceeded intake was largely dependent upon the amount of food eaten. Those mice which ate less drew upon their own tissues to a greater extent.

TABLE 2.—*Effect of the level of dietary protein on the nitrogen balance of albino mice of strain-A-backcross derivation (ABC) bearing a transplant of tumor 15091A*

Group [1]	Mouse No.	Sex	Body weight: Initial	Body weight: Final	Time on experiment	Diet	Total nitrogen intake	Nitrogen output: Urine	Nitrogen output: Feces	Nitrogen output: Total	Nitrogen balance: Total	Nitrogen balance: Daily	Nitrogen in tumor	Wet weight of tumor	Nitrogen in mouse
			Grams	*Grams*	*Days*		*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Grams*	*Milligrams*
	1	F	22.1	11.5	72	N-low	911.7	1,240.0			−328.3	−4.6			
	2	M	20.4	14.6	66	do	860.2	1,075.9			−215.7	−3.3			
1 [2]	3	F	21.2	23.5	66	Adequate	4,527.2	3,749.2			+778.0	+11.8			
			23.5	16.3	6	Fasting	0.0	108.8			−108.8	−18.1			
	4	M	27.1	29.1	63	Adequate	4,729.2	3,924.1			+805.1	+12.8			
			29.1	19.2	6	Fasting	0.0	155.6			−155.6	−25.9			
	5	F	20.8	15.2	30	N-low	212.8	339.9			−127.1	−4.2	77.8	3.4	655.5
2	6	F	22.7	16.0	36	do	459.2	624.2			−165.0	−4.6	20.8	.8	440.0
	7	F	22.0	15.2	36	do	376.7	517.8			−141.1	−3.9	26.8	1.3	433.8
	8	F	19.5	12.4	27	do	239.4	346.5	57.0	403.5	−164.1	−6.1	50.0	1.9	362.0
3	9	F	19.0	13.0	27	do	225.5	289.8	28.9	318.7	−93.2	−3.5	50.0	2.4	391.0
	10	F	20.7	12.9	27	do	218.5	319.0	56.3	375.3	−156.8	−5.8	61.0	2.6	371.0
	11	M	27.9	19.1	20	do	141.7	344.8	65.5	410.3	−268.6	−13.4	46.8	1.9	573.5
4	12	M	28.6	18.4	20	do	185.1	379.0	72.5	451.5	−266.4	−13.3	1.61	<.1	594.5
	13	M	28.6	19.1	20	do	172.8	383.8	58.5	442.3	−269.5	−13.5	23.3	.8	605.0
	14	M	20.8	16.8	16	do	136.7	222.1	36.8	258.9	−122.2	−7.6	35.0	1.8	444.0
5	15	M	18.1	15.5	16	do	137.2	191.0	41.3	232.3	−95.1	−5.9	37.0	1.9	367.0
	16	M	22.0	19.5	16	do	151.3	248.6	45.0	293.6	−142.3	−8.9	48.5	2.4	462.5
	17	M	23.2	15.0	27	do	193.6	304.5	38.3	342.8	−149.2	−5.5	41.2	2.0	445.0
6	18	M	19.1	11.9	43	do	383.9	419.5	69.5	489.0	−105.1	−2.4	27.9	1.2	338.0
	19	M	19.6	12.5	43	do	272.6	377.4	77.5	454.9	−182.3	−4.2	30.4	1.4	328.0
	20	F	22.8	25.4	36	Adequate	2,312.6	1,890.4			+422.2	+11.7	118.0	5.3	495.8
2	21	F	23.4	26.1	36	do	1,909.5	1,573.6			+335.9	+9.3	205.3	9.7	457.8
	22	F	23.9	26.7	36	do	1,728.8	1,430.1			+298.7	+8.3	230.8	9.6	[3]
	23	F	17.8	23.4	35	do	1,988.3	1,452.4	109.0	1,561.4	+426.9	+12.2	118.8	4.6	591.0
3	24	F	17.2	19.6	24	do	1,141.1	957.6	64.3	1,021.9	+119.2	+5.0	126.3	5.8	484.0
	25	F	17.6	23.1	27	do	1,524.6	1,208.4	91.0	1,299.4	+225.2	+8.3	171.3	8.0	464.0
	26	M	25.8	22.7	20	do	820.5	758.0	91.5	849.5	−29.0	−1.5	78.0	3.4	633.5
4	27	M	27.0	24.8	20	do	1,033.7	922.0	90.5	1,012.5	+21.2	+1.1	63.0	2.6	747.0
	28	M	29.3	26.3	20	do	851.4	833.5	79.5	913.0	−61.6	−3.1	79.5	3.3	745.0
	29	M	18.2	22.4	16	do	894.3	614.0	[3]		+280.3	+17.5	45.8	2.0	589.0
5	30	M	21.8	22.4	16	do	894.6	639.6	94.5	734.1	+160.5	+10.0	33.3	1.4	[3]
	31	M	19.2	22.8	16	do	864.5	582.3	183.0	765.3	+99.2	+6.2	38.8	1.7	[3]
	32	M	21.6	17.7	27	do	1,274.2	1,146.9	96.8	1,243.7	+30.5	+1.1	18.5	.6	574.0
6	33	M	21.1	25.1	31	do	1,733.5	1,355.1	143.0	1,498.1	+235.4	+7.6	100.0	4.4	661.0
	34	M	19.0	25.6	40	do	2,405.6	1,726.8	158.8	1,885.6	+520.0	+13.0	130.5	6.0	634.0

[1] Tumor transplants made at the same time into mice of litters which were evenly represented on each diet constitute a single group.
[2] No tumor transplanted.
[3] Sample lost because of foaming during digestion.

Among the mice on the adequate, or 18-percent-casein, diet, the tendency was toward a definite and considerable nitrogen retention. Except in four cases, in mice with tumors, this retention exceeded that found in the tumor. In these four exceptions, the food intake for one reason or another was not normal. In the case of mouse No. 24, the food eaten decreased progressively from the first appearance of the tumor until the animal was killed. However, from the start, mice Nos. 26, 27, and 28 had uncertain appetites which remained unchanged with the development of the tumor. Because they ate irregularly, the urinary nitrogen showed fluctuations. These did not, however, drop low enough following poor eating to compensate for the drop in intake. Thus, even though the nitrogen ingested was high as compared with that of mice on the low-nitrogen diet, the over-all balance tended to be negative. The loss of body weight was in harmony with the analytical findings.

Mouse No. 32 also showed only a very slight positive nitrogen balance. In this case, the trouble was a poor food intake during the first week of the experiment. The weight change during this period was from 21.6 to 16.2 gm. Later the amount of food eaten increased, and the mouse gained while the tumor was growing and reached a final weight of 17.7 gm.

In making a comparison between the final weights of the tumors in mice on the two diets, it must be remembered that only those animals in the same group may be directly considered. While transplants made at the same time were from the same tumor and as nearly as possible of identical size, no attempt was made to keep those made at different times the same. However, the tumors grown in mice fed the low-nitrogen diet were in every case smaller than those in mice fed the 18-percent-casein diet. The extent of this difference was variable, possibly depending upon the amount of tissue used in making the transplant and the characteristics of the particular tumor inoculum. But in these tumors, as in those studied previously, the difference effected by diet was on rate of growth and not on the time necessary for them to become established and just palpable.

The uncertainty of the amount of nitrogen present in the mouse at the start of the experiment made the establishment of a complete accounting of all shifts and changes in nitrogen during the growth of a transplanted tumor impossible. Nevertheless, it seems probable that in mice fed the low-nitrogen diet, the tissues of the host supplied part of the nutrients for the growth of the tumor. This low-nitrogen diet contained no protein as such, and the only material utilizable as protein came from the protein split-products in the liver extract whose purpose was to supply part of the vitamins. It was indicated in the preceding paper that the liver extract probably contained no lysine and histidine and at the level used was deficient in at least isoleucine and threonine. The fact that every mouse on the diet, with or without a tumor transplant, was always in negative nitrogen balance showed that tissue nitrogen was being used even for maintaining life. That under these conditions a tumor was able to grow, demonstrated that in some way the tumor was able to demand and receive the nutrients missing from the diet. The host itself was the only available source of these constituents. Thus it becomes an inescapable conclusion that the tissues of the mouse bearing a tumor transplant are available, if necessary, to meet the needs of the tumor.

SUMMARY AND CONCLUSIONS

Nitrogen-balance studies were made on strain ABC albino mice into which transplants of tumor 15091 A were made. The diet of these mice was either one containing 18-percent casein or one without protein. It was shown that these tumors became established and grew in mice fed the low-nitrogen diet while the host was continuously in negative nitrogen balance. This indicates a strong probability that the tissues of the host, at least under these conditions of stress, are available to the tumor to supply its needs for growth.

A RESTRAINING JACKET FOR THE RAT

By HAROLD P. MORRIS, *senior nutrition chemist*, and THELMA B. DUNN, *research fellow*, *National Cancer Institute, National Institute of Health, United States Public Health Service*

In several types of experiments where rats are used, it is necessary to restrain the animal so that it cannot reach certain parts of the body with the mouth or teeth. This restraint is especially required if one wishes to hold any substance in contact with the skin or an open wound by means of dressings, for the unrestrained rat will usually remove any skin dressing in a few hours' time. It is important also that the restraint used shall interfere as little as possible with the animal's comfort and general health. The devices described in this paper fulfilled these requirements more closely than any other of the numerous measures which were tried.

Jackets following the general designs shown in figure 1, *A* and *B*, were cut from a single piece of "galvanized after weaving" wire mesh or hardware cloth

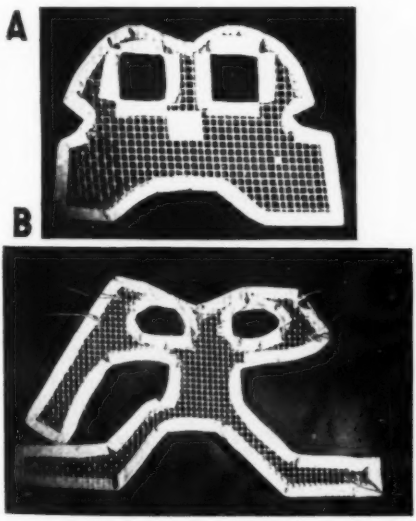

FIGURE 1.—Restraining jacket for the rat. *A*, Design 1; *B*, Design 2.

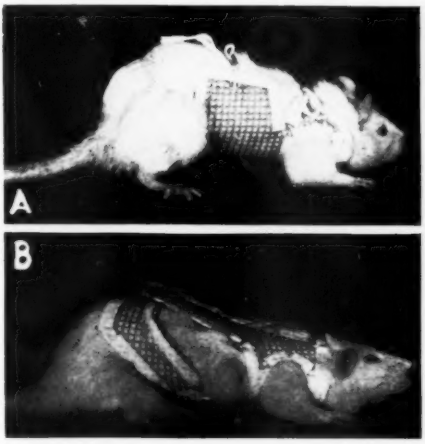

FIGURE 2.—Restraining jacket fitted to the rat. *A*, Design 1; *B*, Design 2.

measuring 8 meshes to the inch. A slight variation in size of the jackets was required, depending on the size of the animal. The edges were then bound with adhesive tape; while the animal was under light ether anesthesia, the jacket was fitted snugly and tied at the neck and across the back with short pieces of twine (fig. 2, *A* and *B*). The design shown in figure 2, *A*, was easily made and adjusted to the animal and was useful for short periods of observation. The second design (fig. 2, *B*), appeared to be more comfortable, and less injury occurred from the edges of the jacket. This was generally used when long periods of observation were required. With either design, the animals were examined each day, and if the edges of the jacket or the twine was causing any injury to the skin, adjustments were made to secure a better fit of the jacket. During the first few days in restraint, nearly all animals showed a slight loss of weight, which was usu-

ally less in the female. This loss, however, was generally regained, and some animals were kept in their jackets as long as 6 weeks.

Advantages of restraints of this type are that the jackets can be made easily and inexpensively, they can be used repeatedly, and they can be worn by the animals for a long period. When properly fitted, the animals do not escape from them, and they are more easily handled for close observation, as under a dissecting microscope for accurate measurements of wounds.

TECHNIQUE FOR STUDYING EPITHELIZATION OF PAIRED SKIN WOUNDS IN RATS, WITH OBSERVATIONS ON THE INFLUENCE OF DIETARY PROTEIN

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INTRODUCTION

One of the broader fields of cancer research involves the study of the factors affecting the growth of normal cells. The healing wound is a readily available means for observing such normal cell growth as it occurs during the process of regeneration. Dietary factors can be readily modified during this process, however. None of the methods heretofore available for the measurement in vivo of epithelial growth in laboratory mammals, as affected by dietary constituents, have proved adequate. The present investigation was prompted by a need for a more precise method of measuring epithelization in a species widely used in dietary experiments and about which a vast amount of knowledge of its nutritional requirements is available. The results are reported of the application of a new technique to a study of the influence of short periods of dietary-protein depletion on the rate of epithelization of skin wounds in rats.

Recently Crandon, Lund, and Dill (*1*) and Bartlett, Jones, and Ryan (*2*) reported that the failure of deposition of collagen in the absence of ascorbic acid results in failure of surgical wounds to heal. Crandon, Lund, and Dill further reported the fact that ascorbic acid must be almost entirely absent from the blood before failure of healing is evident and that this failure occurs only after the absence, for several months, of ascorbic acid in otherwise normal persons. This effect of ascorbic acid deficiency does not appear to be important if the intake has been reduced for short periods only.

On the other hand, a reduction of only 15 percent in plasma protein, according to Thompson, Ravdin, and Frank (*3*), may lead to a moderate delay in wound healing whereas a reduction of 25 percent is severe enough to cause very serious delay or complete failure. If the healing of wounds is so markedly affected by such a moderate lowering of the plasma proteins, would it be possible to influence wound healing either by greatly increasing dietary protein or by reducing the amount of protein from normal? Clark (*4*) in 1919 reported that the latent period of healing was shortened in the surgical skin wounds of dogs fed a high meat diet. These experiments, however, were not extensive or well-controlled. Young, Fisher, and Young (*5*) concluded that the healing of skin wounds in rabbits depended somewhat on general or constitutional factors, although these authors gave no indication that the factors might be nutritional. Weech (*6*) recently was able, in a period of 3 weeks, to cause a measurable depletion of the serum albumin in dogs fed a standard diet low in protein and was able to measure the efficacy of a number of proteins in replenishing the depleted blood proteins. On the assumption that the rat could also be depleted of protein reserves during a short period of feeding on a low-protein diet, it seemed probable that with suitable techniques it would be possible to study the relation of protein intake to the healing of surgical wounds in this species.

MATERIALS AND METHODS

The use of the rat for determining the effect of dietary protein upon the process of epithelial regeneration in the skin necessitated (1) some type of restraining device which would not only prevent the animal from chewing off dressings or licking the healing edges of the wounds but also would cause a minimum of interference with the health and normal activity of the animal, and (2) a means of inhibiting or controlling the closing of the wounds primarily by contraction (the normal process in most mammals).

Several strains of adult rats were used in this study (Wistar, Osborne and Mendel, Sprague-Dawley, and Buffalo (a strain originally obtained by the National Institute of Health in 1930 from the State Institute for Malignant Diseases, Buffalo, N. Y., through the courtesy of Dr. C. F. Cori, and bred at the National Institute of Health since that time primarily for the maintenance of transplants of the Jensen sarcoma).

It has been found in this laboratory that undressed surgical skin wounds in the rat when undisturbed close very rapidly, but with a minimum amount of epithelization, the major portion of the closing appears to be the result of contraction of the skin margins. Obviously, the effect of feeding dietary constituents upon the regeneration of fibroblasts and epithelial cells cannot be adequately determined in such wounds.

Howes (*7*) and Henshaw and Meyer (*8*) sought to eliminate the effect of contraction by placing the wounds on the ears of the rabbit. Contraction is minimal in such a location, because the skin of the rabbit's ear rests only on a small amount of connective tissue surmounting cartilage to which it is closely adherent. Howes further attempted to eliminate the factor of contraction by measuring the growth of epithelium outward from an island of intact skin, 2 to 3 $mm.^2$, at the center of the wound. The lack of adaptability of the rabbit to dietary studies and insufficient laboratory facilities made it essential to use a smaller animal for wound studies. Restraint of the animal was reasonably well provided for by the use of the devices described by Morris and Dunn (*9*). Of the many attempts to control contraction of the wound, two were found to be fairly satisfactory. One procedure, for circular wounds, is described in the present paper, and the other, for rectangular wounds, is described by Dunn (*10*).

The best location for the circular wounds is on the animal's rump. This area appears to be favorable for the greatest degree of immobilization of the skin and aids the attachment of the wound margins to the underlying tissues. This site probably causes the least disturbance of the wound during the normal activity of the rat and also during the handling incident to measuring the wound diameter and replacing the dressing, especially at the fourth day.

The use of the rump also permits the making of bilateral wounds on each animal. This is a desirable feature when topical application of materials is being studied. The control and treated wounds are in comparable locations on the same animal and therefore are subject to the same physiologic influences. Barring infection, such pairing of wounds enhances the efficiency of a statistical analysis of the data for significant differences in treatment.

The protein content of the diet was varied in this study, and therefore the measurements of both wounds were utilized in computing the statistics for any given dietary regimen. The uniformity of healing of the wounds on the right and left sides was studied, and the results are reported herein.

The experiments were designed to study the effect of a low and a very high protein diet on wound healing. An experiment was also carried out to determine the effect of raising the dietary protein to a high level at the time of making the wound in protein-depleted animals. The effect of adding three of the essential amino acids, valine, tryptophane, and lysine, to the low-protein diet at wounding time was studied in a preliminary way.

The composition of the synthetic diets used is given in table 1. Lactalbumin was used as the source of protein because of the observations of Weech (*6*), which indicated that this protein was a much better material for serum-protein regeneration in the dog after depletion by dietary procedures, than is either casein or egg white, and nearly as good as fresh beef serum, the highest on the list of the eight reported by Weech. The diets were fed for at least 2 weeks prior to

wounding in order to put the animals in a uniform nutritive state, especially so far as protein was concerned. Two weeks of feeding a low-protein diet is probably a minimum rather than a maximum period for the depletion of the normal protein reserves in the body of the experimental animal.

TABLE 1.—*Dietary constituents used in wound-healing studies*

Dietary constituents	Diet No. —		
	122	123	124
	Grams	*Grams*	*Grams*
Lactalbumin (Borden technical grade)	7	60	32
Cerelose (corn dextrose)	62	9	37
Lard	10	10	10
Corn oil	10	10	10
Cod-liver oil	2.0	2.0	2.0
Wheat-germ oil	.5	.5	.5
Salt mixture (185a)[1]	4.0	4.0	4.0
Choline hydrochloride	.5	.5	.5
Ryzamin B	4.0	4.0	4.0
Total	100.0	100.0	100.0
	Mg.	*Mg.*	*Mg.*
Riboflavin	1.0	1.0	1.0

[1] See Morris and Robertson (*11*).

EXPERIMENTAL PROCEDURE

WOUNDING TECHNIQUE

The animal was initially anesthetized with sterile veterinary nembutal (Abbott) containing 65 mg. nembutal per cubic centimeter. The dose found most satisfactory was 0.025 cc. containing 1.6 mg. nembutal per 100 gm. body weight. This dose did not induce complete sedation, and ethyl ether was administered by the respiratory route to complete the process during the operation. After partial sedation, the animal's hair was clipped on the entire dorsal vertebral surfaces from the base of the tail over the flanks to about 2 cm. anterior to the crest of the ilium (or disk crest). The clipped area was washed thoroughly with soap and water, dried, and swabbed with acetone and finally with 70 percent alcohol, to remove dirt and grease and to permit a more effective attachment of disks to the skin.

Perforated, sterilized, aluminum disks were first attached to each side of the rump with a thick coating of collodion and further secured by suturing to the skin with silk thread soaked in 70 percent alcohol (fig. 1). These disks were 3.5 cm. in outside diameter and had a 1.9-cm. perforation in the center. The rim, which was 0.8 cm. wide, was perforated with 11 holes each 0.5 cm. in diameter. The holes were cut with a jig, and 6 or 8 disks were cut simultaneously.

In attaching the disks, the animal was first covered with a sterile cloth having a cut-out area just large enough to expose the surgical field. Eleven sutures on the outside edge and five on the inside edge of the disk were made through the skin. The use of collodion in attaching the disk greatly facilitated suturing. A 3/8-circle, size-12-cutting-edge, stainless-steel surgical needle was found most satisfactory for suturing. The knots were touched with collodion to insure their staying in place. It was found that tight sutures resulted in poor circulation and swelling of the tissues both under and around the disk.

After the sutures were in place (fig. 1, *B* and *C*), the collodion seal between skin and the disk was repaired where it had become loosened. The inside of the disk was first swabbed with 70 percent alcohol, then a surgical wound, made with sterile instruments, was prepared by removing the entire epidermis down through the panniculus carnosus. The skin was cut in a circle concentric with the inner circle of the disk but sufficiently small so that a margin of skin approximately 2.5 mm. wide containing the five inner sutures remained between the edge of the wound (fig. 1, *E*) and the inner edge of the disk. The securely attached disk was a very effective means of retarding contraction of the wound margins and permitted a maximum amount of epithelial growth.

Sterilized dressings were used throughout the healing period to pro-

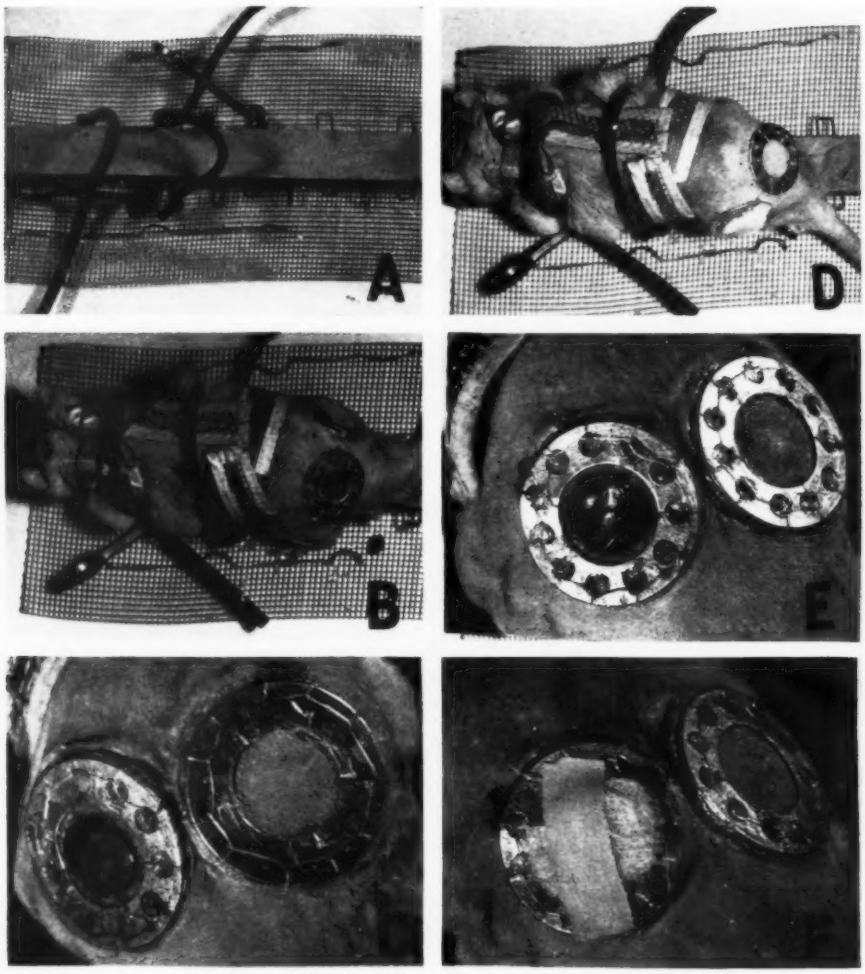

FIGURE 1.—*A*, Holder for rat during examination and measurement of wounds, showing rubber tubing and pinch clamps; *B*, Location of disks; *C*, Enlarged view of disk, showing method of suturing; *D*, Left side of animal with wound covered with a dressing; *E*, Enlarged view of wound, showing margin of skin around inner circle of disk; *F*, Enlarged view of dressed wound shown in *D*.

tect the wounds. A piece of perforated cilkoid (a cellophanelike material which can be sterilized) slightly larger than the initial wound area was placed directly over the wound. This material, which was easily removed without disrupting the healing surfaces, prevented the cotton-dressing pad from sticking to the unhealed tissues yet permitted the maintenace of a dry wound as the exudate could be absorbed by the dry cotton-gauze pads placed directly over the cilkoid. These pads (fig. 1, *D* and *F*) were made from 8 to 12 folds of coarse gauze and were approximately 20 mm. in diameter, and

thus sufficiently large to cover completely the inner edge of the aluminum ring. They were kept in place by a strip of adhesive over the gauze pad which was firmly anchored at each end to the disk, by means of collodion (fig. 1, *D* and *F*).

The wounds were dressed and measured on the fourth, eighth, eleventh, and every day or every other day thereafter until the wounds were covered with epithelium. Fresh dressings like the first ones were used. Extensively infected wounds were discarded, but slightly infected wounds that appeared occasionally were included as they healed within approximately the same length of time as did the uninfected wounds.

During the dressing of the wounds, the animal is held firmly by a simple device (fig. 1, *A*), which consists of a strip of wood $\frac{3}{4}$ x $\frac{3}{4}$ x 8 inches, attached to a wide base. Several staples are driven into the strip at varying distances apart to accommodate rats of different sizes. Two pieces of rubber tubing are fastened to two staples, passed over the animal, drawn through staples on the opposite side, pulled fairly tight, and then clamped with pinch clamps. Both hands of the operator are thus free to measure and to dress the wounds. When the dressing and measuring are completed, the pinch clamps are released, and the animal is immediately freed.

Measurements were taken with a modified caliper, made by permanently attaching to each jaw pointed Bard Parker detachable knife blades. The measurements were usually made under a dissecting microscope for greater accuracy in determining the exact margins of the epithelial overgrowth. The pointed tips were essential for the microscopic observations.

RESULTS

HEALING RATIOS

A total of 100 wounds was used in compiling this report. Several additional wounds were observed, but they were omitted because of infection, failure of the animal-restraining device, failure of the wound restraint, etc.

The healing ratios (complete epithelial closing) were calculated for these wounds by using the formula of Lumière (*12, 13*) in which the rate V is equal to $\frac{L}{T}$, where L is the average diameter of the wound in millimeters (average of two measurements at right angles to each other) and T the time in days required for complete overgrowth of the epithelium. Obviously, with the use of this formula the lag phase is not measured, which in these wounds was probably 4 to 6 days. Since we were interested in the total time for complete epithelial covering, and too frequent changing of the dressings might delay the onset of epithelization (Howes (*7*)), we did not actually measure the latent period. The first measurement, made on the fourth day, was selected as being close to the end of the latent period. Table 2 gives the healing ratios for the wounds.

Higher values for the healing ratios indicate more rapid healing. The average healing ratio for the low-protein group (A) was 0.729, as compared with 0.894 for the very high protein group (B): the difference, 0.165, is statistically very significant. The average ratio for the medium-high-protein diet (C) was 0.895, which is practically the same as that for the diet twice as high in protein. Depleting the protein reserves of the animals for 2 weeks prior to wounding by feeding a low-protein diet and then placing the animals on a high-protein diet on the day of wounding (group D) resulted in a higher average healing ratio than for group A, which was on the low-protein diet for the entire period. The difference, 0.098, was statistically very significant. The addition of the amino acids, valine, tryptophane, and lysine, to the low-protein diet at the time of wounding did not result in any improvement in the healing ratios (table 2), although high values were obtained in one pair of wounds of a rat receiving tryptophane. The high values in this

TABLE 2.—*Rate-of-healing ratios in rat skin wounds as affected by level of dietary protein (lactalbumin) and three amino acids*

Healing ratio in group—						
A	B	C	D	E	F	G
Low-protein (diet 122)	Very high protein (diet 123)	Medium high protein (diet 124)	Low-protein (diet 122) before wounding; medium-high-protein (diet 124) after wounding	Low protein +valine	Low protein + tryptophane	Low protein +lysine
0.633	0.993	0.850	0.718	0.767	0.772	0.756
.617	1.030	1.014	.775	.700	.648	.600
.578	1.050	.940	.929	.627	.920	.548
.759	.776	.933	.993	.645	.979	.794
.750	.789	.824	.957	.726	.657	.619
.824	.771	.914	.950	.863	.652	.838
.690	.794	.853	1.008			
.681	.781	.929	.971			
.605	.950	.800	.880			
.933	.986		.893			
.680	.971		.700			
.711	.927		.711			
.685	.776		.778			
.643	.887		.824			
.750	.957		.763			
.742			.732			
.645			.818			
.737			.783			
.947			.744			
.652			.756			
.789			.761			
.778			.767			
.737			.739			
.742			.812			
.726			.863			
.888			.806			
.778			.800			
.681			.925			
.758			.824			
[1].729	[1].894	[1].895	[1].827	[1].721	[1].771	[1].693

Within-group mean square, $=0.009201$ $\sigma=0.0959$.
Mean difference: Group B−group A=0.165 ($t=5.43$, very significant [2]); C−A=0.166 ($t=4.54$, very significant [2]); D−A=0.098 ($t=12.25$, very significant [2]); A−E=0.008 ($t<.1$, insignificant); F−A=0.042 ($t=1.0$, insignificant); and A−G=0.036 ($t=0.8$, insignificant).

[1] Average.
[2] Would occur by chance less than once in 100 trials.

case may have resulted from a less complete depletion of the protein reserves by the time of wounding. Too few wounds were studied in the amino acid groups to attach any significant weight to the findings.

The average diameters of the wounds on different days after wounding were plotted (fig. 2, *A*). The latent period of healing may be considered as the time before the intersection of the line drawn through the initial to 4-day wound, and the line of average healing rate, or epithelial coverage, which includes all wound-diameter measurements from the eighth day until the wound was completely closed. The latent period thus determined was shorter for both high-protein groups, intermediate for the group shifted from low to high protein at the time of wounding, and longest for the low-protein group. The shifting from low to high protein on the day of wounding appeared to stimulate the rate of epithelization (fig. 2, *A*) so that the wounds of this group actually closed almost as soon as did those of animals receiving the high-protein diets continuously for at least 2 weeks before wounding. (See histologic characterization of these wounds in section on microscopic observations.) The low-protein group (fig. 2, *A*) showed a longer estimated latent period than for any of the other groups and also a longer healing time. Figure 2 shows that there is a linear relation between wound diameter and time during the actively healing period. This relation holds not only for the average for different dietary groups (fig. 2, *A*) but also for the right and left wounds (fig. 2, *B*). The solid and the broken line in figure 2, *B*, each represents the average of 14 wounds. They show the close average similarity between the rate of epithelization in the right and left wounds and further indicate the suitability of this technique for the study of the effect of topical applications on the process of epithelization, where one side is used for the control and the other for the treatment. It would be easy to determine the magnitude of the difference between the control and treated wounds in the same animal which would indicate a significant difference of a healing material under test.

BODY WEIGHT AND FOOD INTAKE

Records were made of the body weight and food intake of four groups of rats. The data are plotted in figure 3. The restraining device previously described was placed on the animals 1 to 2 weeks prior to the making of the

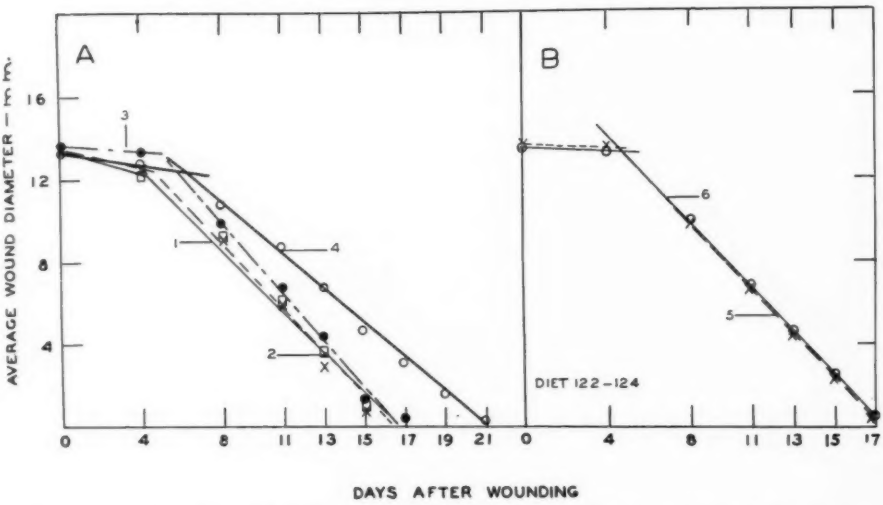

FIGURE 2.—*A*, Average diameters of wounds at varying intervals. 1, Group fed diet containing 60 percent lactalbumin; 2, Group fed diet containing 32 percent lactalbumin; 3, Group fed diet containing 7 percent lactalbumin for 2 weeks before wounding and changed at wounding to diet containing 32 percent lactalbumin; 4, Group fed diet containing 7 percent lactalbumin both before and after wounding. *B*, Average diameter of right and left wounds at varying intervals; 5, Right; 6, Left.

wounds. In each case, the animals lost weight during the period and also there was a greater loss of weight immediately after wounding which was related to the decrease in their food intake on the first day after wounding. Figure 3 shows that all lost weight after wounding except those that were changed to a high-protein diet on the day of wounding. The latter group had a somewhat higher food intake than did the others, although it is not clear from the data that the higher food intake is the only explanation for the difference. However, the animals in the low-protein group showed nearly as high a food intake, yet their healing rate was the lowest of the entire series, which indicates that food intake was not the major factor affecting epithelial growth. The high-protein-diet groups generally ate less food than did the low-protein-diet group yet showed more rapid healing.

MICROSCOPIC OBSERVATIONS

Microscopic sections were made at intervals during the process of healing. In selecting the sections, an attempt was made to choose wounds typical of the various stages; but since the primary purpose of the study was to measure the entire period required for wound closure, a minimum number of animals was used for histologic observation. Even with a small number, it was possible, with a few exceptions, to recognize characteristic and consistent histologic differences in the wounds of animals on a high-protein and on a low-protein diet. Wounds in which characteristic differences were not observed were those of rats on a high-protein diet that refused food or lost excessive weight during the progress of the experiment, and one in which a severe generalized vascular disease was present. All the sections were fixed in Zenker-formol and stained with hema-

toxylin and eosin, after which representative sections were selected from which the photomicrographs were made. Additional stains, as reticulum, Van Gieson, and Mallory aniline blue were also made on the sections selected.

The wounds available for histologic study are listed in table 3.

In the rats on high-protein diet at 4 days (fig. 4, *A*), numerous young fibroblasts, with oval or rounded nuclei, were seen developing at the base of the wound. The intercellular substance was scanty and appeared delicately fibrillar and homogeneous. In the case of the rats on the low-protein diet at 4 days (fig. 4, *B*), all cell nuclei appeared shrunken and deeply stained, and the cytoplasm was scanty and indistinct so that individual cells were identified with difficulty. The intercellular substance contained many coarse fibers, which stained a deep pink with eosin, and red with Van Gieson, and probably represented an unorganized col-

TABLE 3.—*Distribution of wounds studied microscopically*

Wounds of animals on—			Time since wounding
High-protein diet	Low-protein diet	Low to high protein diet [1]	
Number	*Number*	*Number*	*Days*
4	4	.....	4
1	.....	.....	6
5	4	.....	8
4	4	.....	11
2	2	1	13
.....	.....	3	14
2	.....	.....	15
2	.....	.....	16
.....	.....	2	19
.....	3	2	21
2	2	.....	22
2	.....	.....	24
.....	2	.....	25

[1] At time of wounding.

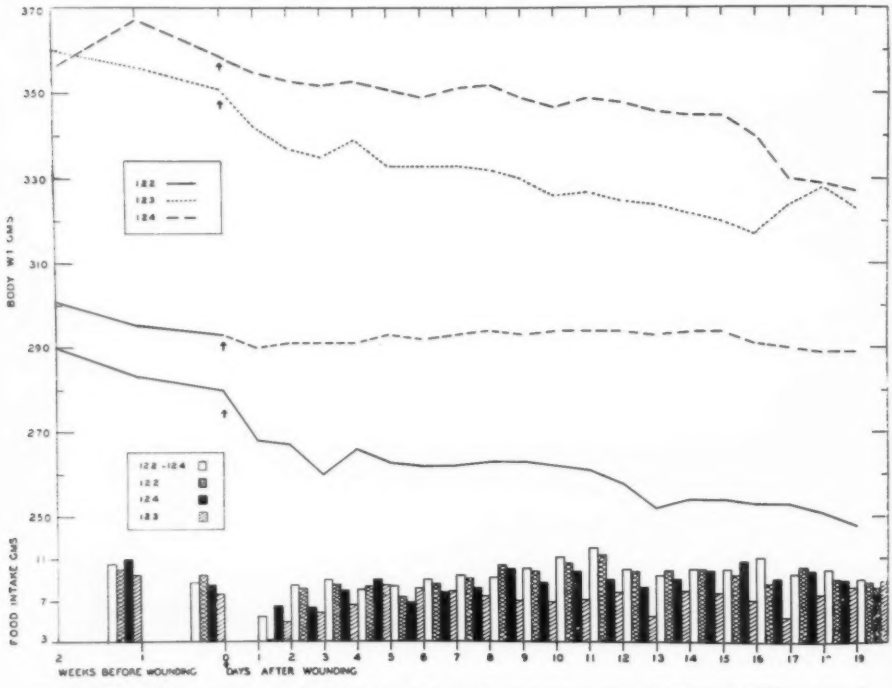

FIGURE 3.—Body weight and food intake of wounds in four groups of rats fed different levels of protein. Rats on diet 122 received 7 percent lactalbumin; those on diet 123, 60 percent, and those on diet 124, 32 percent.

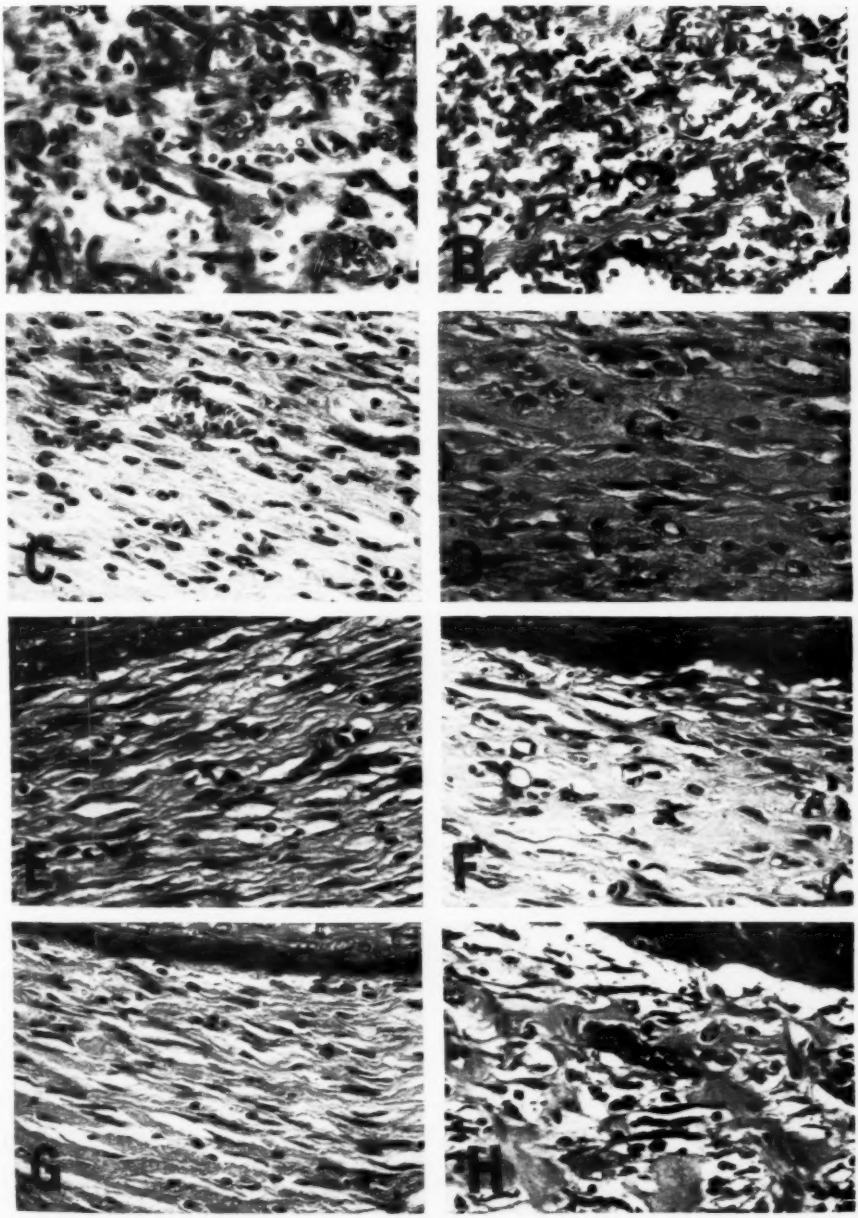

FIGURE 4.—Effect of high- and low-protein diets on wound healing. *A* and *B*, 4-day wounds: *A*, High-protein diet, fibroblasts developing at the base of the wound; *B*, Low-protein diet, intercellular spaces, collagenous debris, and shrunken nuclei apparent; *C* and *D*, 8-day wounds; *C*, High-protein diet, orderly arrangement of fibroblasts, with small amount of fibrillar intercellular substance evident; *D*, Low-protein diet, excessive amount of fibrillar and granular intercellular substance and relatively few cells may be noted. *E* and *F*, 13-day wounds; *E*, High-protein diet, maturing fibroblasts with intercellular collagenous fibrils shown; *F*, Low-protein diet, excessive intercellular substance and tissue spaces evident. *G*, 24-day wound, high-protein diet, shows normal healing, with mature fibrocytes, and well-organized connective tissue scar. *H*, 21-day wound, low-protein diet, numerous intercellular spaces and nonfibrillar intercellular substance apparent. Hematoxylin and eosin strain used for all. × 310

lagenous debris. Intercellular spaces, sometimes crossed by a fine network of fibers, were especially numerous at this stage and were probably the result of the marked edema of the tissue. Their appearance suggested the excessive shrinkage which may occur from improper fixation; but this condition was seen only in the rats on a low-protein diet, and all wound tissues were prepared in the same manner.

Wounds in the high-protein group at 8 days (fig. 4, *C*) contained abundant fibroblasts with elongate nuclei, which were in orderly alignment, with a moderate amount of a rather delicate, fibrillar, intercellular substance that appeared evenly distributed between the cells. The wounds in the low-protein group at 8 days (fig. 4, *D*), on the other hand, contained fewer fibroblasts, and the nuclei often appeared shrunken and pyknotic. Between these cells in many areas were masses of a granular substance which took the stains for collagen. Intercellular spaces, probably owing to edema, and similar to the condition at 4 days, were seen frequently.

The differences in the wounds at 13 days (fig. 4, *E* and *F*) were similar to those observed at 8 days. The intercellular fibrils in the wound of the rat on the high-protein diet (fig. 4, *E*) were coarser than those at 8 days, and a considerable part of the wound was covered with epithelium.

At 24 days, healing in the wound of the high-protein animal (fig. 4, *G*) was complete. Heavy collagenous fibers lay in orderly arrangement between the elongated and shrunken fibroblast nuclei, which at this time resembled the usual fibrocyte of adult connective tissue. The wounds of the low-protein animal at 21 days (fig. 4, *H*) on the contrary did not show such successful restitution of the connective tissue (although they might have been covered with epithelium). Many of the fibroblast nuclei were ovoid, and interstitial edema, as evidenced by intercellular spaces, was striking. Masses of a finely granular, eosinophilic substance were also seen. Collagenous fibrils which had formed in some parts of these wounds were more delicate than the fibers formed in wounds of the high-protein-fed animals.

Healing wounds in animals on a low-protein followed by a high-protein diet beginning at the time the wound was made showed many fibroblasts with ovoid nuclei as long as 19 to 21 days after wounding and a more abundant, granular, poorly organized, intercellular substance than was seen in animals on the high-protein diet. However, the intercellular spaces which were observed in animals on the low-protein diet were not characteristic of these wounds. It seems probable that the osmotic disturbance that accompanies a hypoproteinemia is quickly corrected by adequate protein intake, while the production of new fibroblasts takes place more slowly.

DISCUSSION

The experiments were originally planned to determine the practical value of feeding a high-protein diet to rats during the healing of surgical skin wounds. Since no adequate technique was available that could be applied to the study of epithelial wound healing in rats, it was necessary to devise one that would have good reproducibility.

The use of the restraining device (*9*) for the rat and a method of at least partially controlling the closing of skin wounds by contraction described herein finally resulted in a technique that can be used (1) to study the effect of dietary constituents on the healing of skin wounds in the rat and (2) to study the effect of the topical applications of materials thought to have effect on the healing process.

The excellent reproducibility of the average rate of healing in the right and left wounds on the same animal should make it possible to apply this technique, with greater precision than has been possible heretofore, to various problems involving healing ointments or other topically applied materials.

The studies of wound healing were discontinued before such tests could be

merous intercellular spaces and nonfibrillar intercellular substance apparent. Hematoxylin and eosin strain used for all. × 310

made: for that reason, a detailed description is given of our most successful technique so that our experience can be made available to other investigators that may be interested in further studies on this subject.

It is not surprising that the growth of epithelial cells in a wound could be influenced by dietary protein since the synthesis of protein is an integral part of any new cell growth. If the normal protein reserves are depleted, then the normal reaction of new cell formation to the stimulus of the wounded tissue would not be elicited because the dietary components necessary for the synthesis of the proteins of the new cells would be less available.

No measurement was made of the actual amount of depletion in mobile protein reserves which occurred in the animals fed a low-protein diet 2 or more weeks. Such a short period of feeding such a diet is probably a minimal period in which to obtain a maximum abnormal or delayed healing response. The fact that an inhibition in rate of epithelization was measurable grossly indicates that skin-wound healing in rats is very responsive to low dietary protein. Further studies could show whether this is an effect of specific amino acids or is the result of a depletion of the entire protein moiety. Different proteins may differ in their power to affect wound healing. The preliminary studies using three different amino acids essential for growth in the rat were entirely negative.

Rhoads, Fliegelman, and Panzer (*14*) suggested that hypoproteinemia induced in dogs with repeated plasmapheresis delayed fibroplasia in wounds by first interfering with the osmotic relationship between plasma and tissues. This condition in turn would decrease the amount of protein that could be withdrawn from the plasma for use by the tissues for protein repair. Microscopic examination of the wounds in rats on a low-protein diet would seem to support such hypotheses, because of the excessive edema, poor organization, tardy development of fibroblasts and mature collagen. Microscopic examination of the wounds also supports the explanation of Thompson, Ravdin, and Frank (*3*) that hypoproteinemia is an important cause of wound disruption in dogs even though epithelization appears from gross observation to be complete.

SUMMARY

One hundred surgical skin wounds in the rat were studied by using a new technique which retards and partially controls closing of the wound by contraction.

The technique gave essentially identical average rates of healing in wounds on the right and the left side of the same animal. The average rate of closing of circular wounds after the initial lag period was linear.

The most rapid rate of epithelization was attained in groups on high-protein diets. A significantly lower rate of healing was obtained in animals fed a low-protein diet. Wounds in a group fed a low-protein diet 2 weeks prior to wounding and then changed to a high-protein diet at the time of wounding showed as rapid epithelization as did groups not depleted in protein prior to wounding.

The estimated lag periods of healing were shortest for the high-protein group, intermediate for the low-plus-high-protein group, and longest for the group on low-protein diet.

A loss in body weight after wounding was noted in all groups except those changed to the high-protein diet after wounding.

The addition, at the time the wound was made, of any one of three essential amino acids, valine, tryptophane, and lysine, to the low-protein diet had no beneficial effect on retarded epithelization.

Histologically, the wounds on rats receiving the low-protein diet were distinguished from those on animals on a high-protein diet by excessive edema, poor organization, a deposition of a granular intercellular substance, and tardy development of fibroblasts and mature collagen fibers.

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TECHNIQUE FOR MAKING AND EXAMINING EXPERIMENTAL EPITHELIAL WOUNDS IN THE RAT

By THELMA B. DUNN, *research fellow, National Cancer Institute, National Institute of Health, United States Public Health Service*

Two requirements for observations on the process of epithelial regeneration in the skin of the rat were found to be (1) some means of restraining the animal so that the wound and dressing would not be disturbed, and (2) some means of preventing the usual closure by contraction in a superficial skin wound (the normal process in most mammals), so that an appreciable regeneration of fibroblasts and epithelial cells could be examined or measured. The first of these requirements was fulfilled by the restraining jacket described on pages 269 to 270 of this Journal; and while no entirely satisfactory solution was found for the second problem, the method described herein appeared to be the most practicable.

FIGURE 1.—Experimental wound in rat with dressing in place.

Adult rats were fitted with restraining jackets, and an area on the rump about 2 inches square was shaved with an electric clipper and cleaned with soap and water followed by 95 percent alcohol. The animal was lightly anesthetized with ether, and the shaved area was covered with a piece of muslin which had been dipped in rather thick collodion. The muslin was allowed to become nearly dry before the outline of a wound of definite size was marked out with a soft pencil. The skin down through the panniculus carnosus was cut through with fine sharp scissors and the skin segment lifted out. Any substance to be tested for its effect on regenerating cells could then be applied to the wound surface, which was next covered with a small square of sterilized cilkloid (a perforated transparent dressing resembling cellophane), several thicknesses of sterile gauze, and finally with a square of muslin which was attached, by means of collodion, along the edges of the first piece of muslin (fig. 1). If desired, bilateral wounds of the same size could be made, and one used as a control. Adhesive plaster was sometimes used in place of the muslin and collodion and appeared to be equally satisfactory. When it was used, the final covering of muslin was attached by fine adhesive strips.

It was found that in the normal process of healing of a rat skin wound the edges of even large wounds were soon approximated, and only small linear scars remained. A collodion or adhesive dressing, however, held the skin edges apart so that there was some new formation of epithelial cells. If the dressings were changed or tampered with, the edges frequently became loosened after a few days, and the normal closing by contraction was observed. For this reason the wounds were usually left undisturbed until the animals were killed, at which time the amount of epithelium covering the wounds was measured. Microscopic sections were then made to determine cellular changes. The dressing usually became loose between the sixteenth and the twenty-first day, and by this time the wound might be completely healed. It was found that great variability existed in the rate of healing in all wounds and

that this variability was usually the result of contraction rather than tissue regeneration. It was therefore considered necessary to make histologic examinations of undisturbed wounds at fixed intervals in order to determine what degree of tissue regeneration had actually taken place.

Figure 2 is a photomicrograph of a healed wound in which a considerable area has been covered by regenerated epithelium.

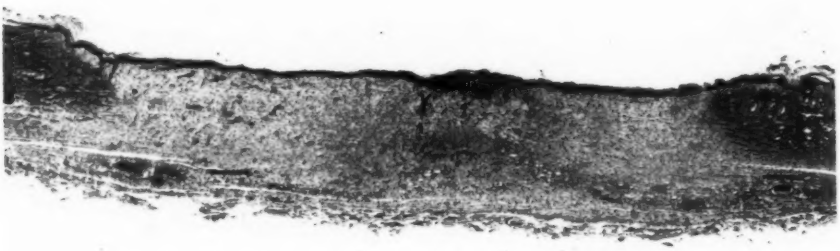

FIGURE 2.—Experimental wound in rat. Healing complete. × 10.

BEHAVIOR OF THYMUS TISSUE TRANSPLANTED TO A SKIN WOUND

By THELMA B. DUNN, *research fellow, National Cancer Institute, National Institute of Health, United States Public Health Service*

In the course of experiments in which the effects of various substances on a healing epithelial wound in the rat were tested, a transformation of thymus tissue from a predominantly lymphatic to an epidermal structure was observed. Because it has been suggested that the small lymphocyte may have a function in the formation of fibrocytes and collagen fibers, thymus tissue, in which this cell is very abundant, was taken from rats 3 to 6 weeks old and applied to the surface of open wounds made on adult rats of the same strain. The method of making these wounds and of applying the material is described elsewhere in this journal (*1*, *2*).

The rats were killed, and the wounds examined and sectioned microscopically following periods ranging from 5 days to complete healing at 18 days or more. In a small percentage of the wounds, the thymus appeared to have taken as a graft, and the epithelial nature of the thymus reticulum was clearly demonstrated. There was no evidence that the usual course of healing was hastened, and no systemic effect on the animal was observed.

MICROSCOPIC OBSERVATIONS

Two wounds examined at 5 days showed degenerating masses of thymus tissue still attached to the wound surface (fig. 1). Fibroblastic proliferation was active in the depth of the wound, and considerable vascularization had occurred. Lying between this regenerating tissue and the thymus tissue remnant was a more compact layer of cells composed of epitheliallike cells with relatively large nuclei and a dense cytoplasm which took the eosin stain. These large cells were heavily infiltrated with small cells morphologically resembling the small lymphocyte. This layer of cells appeared well-vascularized, and many blood-filled channels were seen. There was a clear line of demarcation between this epithelial cell layer and the underlying newly forming connective tissue.

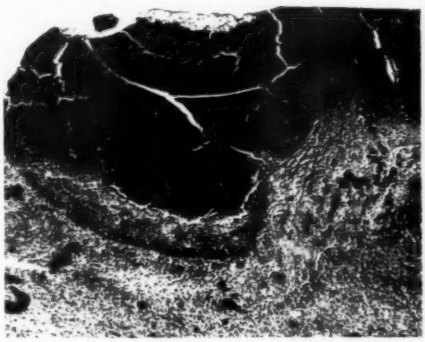

FIGURE 1.—Five-day wound with thymus tissue applied. Layer of epitheliallike cells between regenerating connective tissue and remaining thymus tissue. Hematoxylin and eosin stain. × 16.

One wound examined and sectioned at 8 days illustrated most clearly the alteration which had taken place. When the wound dressing and attached necrotic thymus tissue were removed, the surface below the thymus implant appeared to be covered with a white, opaque membrane, which was closely adherent to the underlying connective tissue. On microscopic examination, the connective tissue appeared to be regenerating normally, for it was well vascularized and contained many young fibroblastic forms. Along the surface was a layer of cells (fig. 2 *A*), whose resemblance to an epidermal structure was evident (fig. 2, *B*). The cells in contact with the connective tissue were smaller and more closely packed, a few were found in mitosis, and the rounded nuclei and heavily stained cytoplasm were characteristic of epithelial cells. With high magnification, intercellular bridges were readily identified in cells lying above this basal layer. In some areas a differentiation toward more flattened cells occurred on the surface,

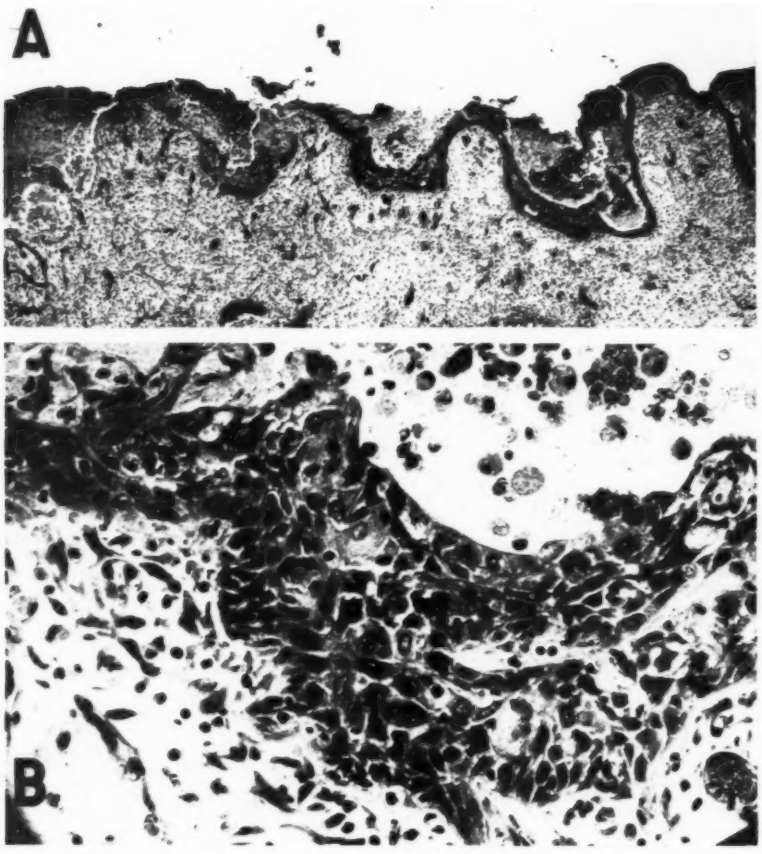

FIGURE 2.—Eight-day wound. *A*. Epithelial cell surface developing from transplanted thymus tissue. × 36; *B*. Higher magnification from *A*. Epidermal cell layers and prickle cells can be identified. × 560. Hematoxylin and eosin stain used in both *A* and *B*.

but no keratinization was observed. Very few blood-filled channels remained among these epithelial cells, but a few endothelial-lined spaces suggested that some vessels had been obliterated. A few lymphocytes were still present. These epithelial cells derived from the thymus differed from the adjacent regenerating epidermal cells, since the nuclei were larger and more hyperchromatic, and they sometimes suggested neoplastic cell forms. Another wound examined at 9 days showed a similar membranous covering, and clearly differentiated epithelial cells were found microscopically, which grew in a sheet over the surface of the granulation tissue bed (fig. 3).

The fate of this thymus epithelial tissue was not definitely determined. In early stages it was separated from the wound margin; but as healing continued, the epidermal cells from the margin of the wound appeared to grow over, intermingle with, and replace the thymus-derived tissue. Whether any cells persisted and actually formed a part of the new epidermal covering

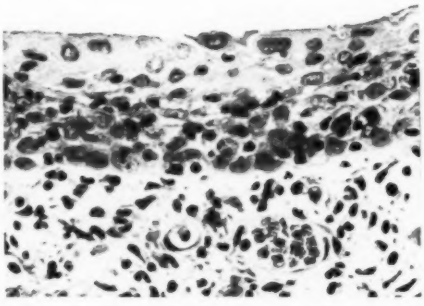

FIGURE 3.—Nine-day wound. Epithelial cells developed on surface from thymus reticulum. Hematoxylin and eosin stain. × 560.

was not ascertained. In the completely healed wounds, areas where the epithelium extended downward into the connective tissue were not unusual, but this was the only difference observed in these thymus-treated wounds and the many others examined.

DISCUSSION

Although the observations on the development of these transformed cells seem conclusive as to the origin of the cells, other possibilities, such as a change in the host epithelial cells or host fibroblasts, were considered. In the many sections of other regenerating skin surfaces examined, no such epithelial formation was ever observed, and these thymus epithelial areas in the early stages were isolated from the epithelial cells of the margin. Thymus tissue from rabbit and mouse was also applied to these rat wounds and was never found to give rise to epithelial cells of this type, even when applied repeatedly to the same open-wound area.

Moreover, other experimental evidence and observations are in favor of considering these cells as derived from the thymus epithelial reticulum. Gottesman and Jaffé (*3*), studying autoplastic thymus transplantation in the guinea pig, observed a destruction of the small thymus cells and hypertrophy and hyperplasia of the reticular cells which led at an early period after transplantation to an almost complete replacement by these cells and an epitheloid appearance of the transplant. In a later paper, Jaffé (*4*) described the appearance of typical Hassall's bodies from these reticulum epithelial cells. Regeneration was usually complete in about 3 weeks, when numerous round cells were again found in the graft, and differentiation into cortical and medullary zones had taken place. An experiment by Foulds (*5*), however, gives the clearest demonstration of the epithelial nature of the thymus reticulum cell. During a study of secondary growths of filtrable tumors of fowls, occurring in the thymus, he observed a concurrent proliferation and abnormal differentiation of the epithelial cells of the gland. His observations raised the problem of the capacity for differentiation of normal thymus tissue which was investigated by autoplastic transplantation in the fowl. He found that the epithelial cells survive and undergo extensive hyperplasia. In later stages some transplants closely resembled the normal gland, while in others there was differentiation of squamous epithelium in various degree. In his conclusions, he stated that it was evident that the potentialities of thymic epithelium were completely realized only under abnormal conditions. Recently Ross and Korenchevsky (*6*) reported that the injection of male or female hormones into the gonadectomized rat caused a disappearance of the small thymic cells, and with estrogenic hormones a specific hyperplasia of epithelial cystic structures occurred. Steiner (*7*) after implantation of methylcholanthrene pellets in the thymus gland of young guinea pigs induced hyperplasia of Hassall's bodies and of the thymic reticulum near the pellet, squamous epithelization of the pellet space, small epithelial cysts, and degeneration of the small thymocytes. These new epithelia showed intercellular bridges and keratohyaline formation. Steiner considered that these structures arose from

Hassall's bodies and therefore showed the epithelial nature of the latter.

Hammar (*8*) and Kingsbury (*9*) summarize their conception of the nature of the thymus reticular cells. In an editorial summary Hammar's opinion is given as follows:

> The thymus gland is fundamentally an epithelial organ infiltrated with lymphocytes. The epithelial components in various mammals are of diverse origin—entodermal in some, ectodermal in some, and combined ecto-entodermal in others.

Kingsbury (*9*) in his interpretation of thymus bodies states:

> The thymus expresses fundamentally an epithelium which has lost its surface value but retains its growth potentiality and also its powers of differentiation which it typically expresses as a degree of keratinization. In such a surface epithelium the differentiation proceeds from the depths (connective tissue contact) toward the free surface; in the thymus lobule the differential growth becomes centripetal, with cell conglomerates forming centrally as expressions of a differential growth in a confined space. Thymic corpuscles are thus similar in formation and structure to the epithelial pearls of a keratinizing epithelioma.

Recognition of such potentialities of the thymus epithelial cells offers a ready explanation for the origin of epithelial tumors from the thymus. One such tumor, as reported by Wu (*10*), even contained isolated prickle cells in small numbers. This behavior of thymus tissue also furnishes another instance of the remarkable variations which cells may undergo under different environmental condition, and again raises the question of the possible role which the small thymus cell, or lymphocyte, may have in affecting the growth of other cell forms.

SUMMARY

A stratified squamous epithelium was developed on the surface of skin wounds in old rats when the wounds were covered by thymus tissue from younger animals of the same strain. This epithelium was derived from the thymus recticulum cells. Such a change in the thymus tissue is not surprising when other evidence on the potentialities of the thymus reticular cell is reviewed.

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GENE—MILK AGENT RELATIONSHIP IN MAMMARY-TUMOR DEVELOPMENT

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INTRODUCTION

It has been conclusively demonstrated that the development of mammary tumor in the mouse is influenced by genetic factors. The problem that follows is to ascertain the number of these factors and especially the physiologic mechanisms through which their effects become manifest.

From crosses between high-tumor-strain A and low-tumor-strain C57 black, Bittner (*1*) in 1940 concluded that susceptibility to mammary-tumor development is inherited and gave, with reservation, a single-dominant-factor interpretation to the data. The location and mechanism of the genic action in controlling this susceptibility was not indicated, but it might be expected that the primary gene action occurred in the mammary-tissue cell. Later Bittner (*2*) indicated that in the problem as a whole multiple genetic factors are involved. That a part of the genetic action on mammary-tumor development is manifest through the hormonal mechanism was indicated by the experiments of Bittner et al. (*3*) and of Heston and Andervont (*4*). The difference between the condition in strain A, in which the virgin females have a low mammary-tumor incidence although that of the breeders is high, and the condition in strain C3H, in which both the virgins and breeders have a high incidence, was shown to be a genetic difference. Another possible mechanism through which genic action could be manifested in tumor development was suggested in the discovery of the extrachromosomal factor, or milk agent.

Murray (*5*) suggested that differences in mammary-tumor incidence of the various inbred strains is owing in part to variability of the extrachromosomal factor, but he did not offer an explanation of the cause of this variability. Little and Gorer (*6*) postulated that if the agent is a virus there may possibly be genetic variability of the agent. De Ome (*7*) stated that the low tumor incidence of strain C57 black may result from the absence of the milk-borne factor or the constitutional inability of strain C57 black to produce such a substance. The data most pertinent to the question were those of Murray and Little (*8*) from an experiment in which they made repeated backcrosses to the resistant-strain male from an original cross of susceptible-strain female to resistant-strain male. By the eighth backcross generation, in which a resistant-strain genetic constitution had been concentrated as a background for the milk agent, it was found that not only did most of the females fail to develop tumors but also that the milk agent had been eliminated since these females did not transmit the agent to genetically susceptible young. Such results indicated that the concentration of resistant-strain chromatin had rendered these females incapable of propagating and transmitting the milk agent.

The present paper reports the results of experiments outlined to analyze this relationship in mice between the genetic constitution and the milk agent, especially the influence of the genetic constitution on the propagation and transmission of the agent.

EXPERIMENTAL PROCEDURE

The answer to the question whether or not the genetic constitution has an influence on the ability to propagate and transmit the milk agent could be obtained only through the observation of the transmission of the agent under conditions where the genetic constitution was varied while all extrachromosomal factors were kept constant. These conditions were fulfilled through the hybridization and foster-nursing procedures shown in figure 1.

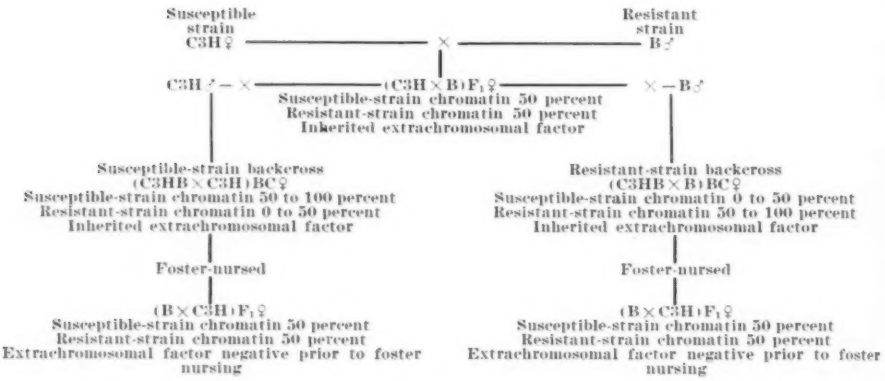

FIGURE 1.—Outline of experiment demonstrating influence of genetic constitution on propagation and transmission of milk agent.

The initial step was the mating of 14 high-mammary-tumor-strain C3H females to low-mammary-tumor-strain C57 black [1] males. Both groups were of sublines of these strains maintained at the National Cancer Institute. The strain C3H subline was originated from animals received in 1930 from Dr. L. C. Strong, and the C57 black subline was originated from mice received in 1939 from Dr. W. S. Murray. Brother-sister inbreeding has been practiced throughout the life of both of these strains. The history of their development has been reviewed in a number of publications (*9–13*), and both have been used extensively in cancer research as high- and low-mammary-tumor strains.

From this original mating of C3H females × C57 black males, 82 (C3H × B)F_1 hybrid females were reared. Since the parental strains were highly inbred, these F_1 hybrid females could be considered as closely approximating genetic homogeneity although 50 percent of their chromatin was derived from their susceptible-strain parent and 50 percent from their resistant-strain parent. As all these F_1 hybrid females came from highly inbred females of the same susceptible strain, it can be asumed that the milk agent and the possible intra-uterine and cytoplasmic influences which they received were highly uniform. In view of their genetic uniformity and the uniformity of the extrachromosomal influences which they had received, together with the fact that they were genetically susceptible to mammary-tumor development, it seemed safe to assume that all of them would transmit the extrachromosomal factors equally.

As the next procedure, 52 of these (C3H×B)F_1 females were backcrossed to males of each parent strain, the remaining 32 being retained as virgins. From the backcross to the high-tumor-strain C3H males, 88 (C3HB×C3H)BC females were reared, and from the backcross to the low-tumor-strain C57 black, or B, males, 98 (C3HB×B)BC females were reared. On the assumption generally made that extrachromosomal factors are probably not introduced with the sperm, one can consider that these two groups of females had received comparable extrachromosomal factors. Their mothers had been of the same uniform group, and thus the milk agent, and the intra-uterine and cytoplasmic influences that both groups had received were comparable. The two groups, however, were unlike in their chromosomal factors. The (C3HB×C3H)BC females with high-tumor-strain fathers

[1] Strain C57 black has often been referred to as strain B, and this designation is used throughout this paper for brevity in distinguishing the different types of hybrids.

contained an average of 75 percent high-tumor-strain chromatin, whereas the (C3HB×B)BC females with low-tumor-strain fathers contained on the average only 25 percent high-tumor-strain chromatin. Segregation of genes, however, resulted in variations within each group, the theoretical limits of high-tumor-strain chromatin of the (C3HB×C3H)BC being 50 and 100 percent, and of the (C3HB×B)BC being 0 and 50 percent. With the genetic constitution varied and all other factors controlled, it was now possible to ascertain whether or not the variation in the genetic constitution would influence the propagation and transmission of the milk agent. The situation also provided an excellent opportunity for evaluating the influence of the genetic variation alone on mammary-tumor development. Any difference in mammary-tumor development of these two groups would be solely a genetic difference.

The ability of these backcross females to transmit the milk agent could be ascertained only through the observation of the mammary-tumor development in suitable test females that had received the milk agent from them. To be suitable, the test females had to be uniform both as to genetic and extrachromosomal factors. It was essential that they did not receive the milk agent before being used as test animals but that they be genetically susceptible to it. It was obvious that the progeny of the backcross females would not be suitable, since they would be variable in both their chromosomal and extrachromosomal factors.

Adequate test females were obtained by mating low-tumor-strain C57 black females to high-tumor-strain C3H males. The (B×C3H)F_1 hybrid females thus produced were genetically uniform. That their mothers were of the same inbred strain contributed toward uniformity of the cytoplasm of the egg from which they developed, uniformity of intra-uterine environment, and uniformity of any milk they received. Since their mothers were of the low-tumor strain, they could not receive the milk agent from their mothers, but since their fathers were of the high-tumor strain, they contained 50-percent-susceptible-strain chromatin and were thus genetically susceptible to the agent. To minimize possible variation owing to litter number, all the test females were of the first and second litters of the C57 black mothers.

At the time that the C57 females were mated to the C3H males to produce the test females, the females of both backcross groups were mated to C57 black males. In this case, the C57 black male was selected principally because of convenience. When each backcross female gave birth to her litter, the litter was discarded, and five test (B×C3H)F_1 females were placed with her. As far as possible, each test litter was divided between females of the two backcross groups, thus supplying litter-mate controls for the test. For the most part, the foster nursing was begun on the day the backcross female gave birth to her litter; in some cases, however, it was begun on the day following, in a few cases, it was on the second day, in one on the third day, and in two on the fourth. Within these limits, however, there was no apparent influence of the length of time following birth of the litter, at which the foster nursing began. The test (B×C3H)F_1 females were placed with the backcross foster mothers usually on the day the test females were born; in a few instances, it was the day following birth, the second, or even the third day after birth, but this variation in age at which the foster nursing began was not reflected in the tumor development in the test females.

Of the total of 88 susceptible-strain backcross (C3HB×C3H)BC females, 78 were tested with a total of 375 foster-nursed (B×C3H)F_1 test females. Four lost their test young, and 6 were permitted to rear their own young, since there were no test females available at the time their litters were born and it was essential that they rear a litter to be comparable with the other females of the group.

Of the 98 resistant-strain backcross (C3HB×B)BC females, 77 were tested with a total of 355 foster-nursed (B×C3H)F_1 test females. Five of these backcross females lost their test young, and 16 were permitted to rear their own young for the same reason as that given for the females of the other backcross group.

The test (B×C3H)F_1 females were permitted to nurse upon their backcross foster mothers throughout the normal nursing period. At 4 weeks of age, they were weaned and set aside as virgins for the observation of mammary-tumor development.

The 6 (C3HB×C3H)BC females that were permitted to rear their own young produced 18 female offspring that were designated as (C3HB×C3H)BC×B since they had C57 black fathers. It should be noted that such females would contain on the average three-eighths C3H chromatin, the theoretical limits being 0 and 50 percent. The 16 (C3HB×B)BC females that were permitted to rear their own young produced 45 female offspring. Since these backcross mothers also were mated to C57 black males, this mating constituted the second backcross, and the offspring were designated as (C3HB×B)BC_2. Such offspring would contain on the average one-eighth C3H chromatin, the theoretical limits again being 0 and 50 percent. These two groups of females, (C3HB×C3H)BC×B and (C3HB×B)BC_2, also were set aside as virgins for the observation of tumor development.

Throughout the experiment, all groups of females were housed in wooden cages, eight animals to the cage, except for the breeding females at the time of mating when they were kept four in a cage with one male and were isolated when pregnant. All were fed Purina dog chow and were given a constant supply of tap water.

Weekly examinations for tumors were made in all groups. Following the gross observation of a tumor, the mouse was necropsied and a histologic diagnosis was made of the tumor. Nontumorous mice were necropsied after they were observed to be moribund or when found dead.

RESULTS

TUMOR DEVELOPMENT IN EACH GROUP

Parental C3H females (Breeders).—Of the 14 females of this group, 13, or 92.86 percent, developed mammary tumors at an average age of 9.62 months, the remaining female dying of pneumonia at 11 months of age. No other neoplasms were found in the group. Although the number of females in this group is not large, the percentage incidence corresponds to that which Andervont (*1*) has published for the strain. In this subline, he found that of 1,588 breeding females, 91.37 percent developed spontaneous mammary-gland tumors at an average age of 8.58 months, and of 350 virgin females, 97.43 percent developed spontaneous mammary tumors at an average age of 10.43 months.

C57 black females.—There were 146 females of this strain used for producing the (B×C3H)F_1 test females. Of these, 94 were later used for other purposes, but of the remaining 52, only 1 developed a mammary tumor, which appeared at 23 months and was of an unusual keratinizing type. The remaining 51 females died at an average age of 19.06 months. This incidence is in accord with others published for the strain. From reports of Cloudman et al. (*15*) and Little (*16*) from 10 to 20 percent of the strain C57 black can be expected to develop nonepithelial tumors, principally of the leukemia group.

(C3H×B)F_1 (Breeders).—Of the 52 females of this group, 50, or 96.15 percent, developed mammary tumors at an average age of 12.86 months. One of those that had a mammary tumor at 6 months also had leukemia with marked thymic enlargement. One of the nonmammary-tumor females was necropsied at 22 months of age, following the palpation of a subcutaneous mass, which was diagnosed histologically as a nonneoplastic cyst. The other nonmammary-tumor female came to necropsy at 28 months with leukemia.

(C3H×B)F_1 (Virgins).—Of the 30 females of this group, 27, or 90 percent, developed mammary tumors at an average age of 14.19 months. One of these mammary-tumor females, necropsied at 19 months, also had developed leukemia, as had another necropsied at 30 months. One came to necropsy at 24 months with leukemia but without a mammary tumor, and 2 others came to necropsy nontumorous at 13 and 30 months of age.

(C3HB×C3H)BC (Breeders).—Of the 88 females of this group, 82, or 93.18 percent, developed mammary tumors at an average age of 11.63 months. One of those that developed a mammary tumor at 13 months also had leukemia, and a second with leukemia

was necropsied at 28 months without a mammary tumor. Five other females came to necropsy nontumorous.

(C3HB×B)BC (Breeders).—Of the 98 females of this group, 54, or 55.1 percent, developed mammary tumors at an average age of 16.61 months. Two of these mammary-tumor females also had leukemia, 1 had a hepatoma, and 1 had an adenoma of the Harderian gland. Eighteen other cases of leukemia were found in this group, and the remaining 26 females came to necropsy nontumorous.

(C3HB×C3H)BC×B (Virgins).—There were but 18 of these females; 11, or 61.11 percent, developed mammary tumors at an average age of 16 months. Of the remaining 7 females, 1 came to necropsy nontumorous, 4 developed leukemia, and 2 developed sarcomas, of which 1 was an osteogenic sarcoma.

(C3HB×B)BC (Virgins).—There were 45 of these females; 12, or 26.67 percent, developed mammary tumors. These tumors appeared when the animals were the average age of 17.17 months. Of the remaining 33, 13 developed leukemia, and the other 20 came to necropsy without tumors.

(B×C3H)F_1 (Virgins) foster-nursed by (C3HB×C3H) BC (Test series).—There was a total of 375 of these females, and 336, or 89.6 percent, developed mammary tumors at an average age of 14.87 months. Aside from the mammary tumors, in the whole group there were 13 cases of leukemia, 5 animals with hepatomas, 2 with pulmonary tumors, 2 with sarcomas, 1 with an epidermoid carcinoma, and 1 with an adenoma of the Harderian gland. Twenty-five came to necropsy without tumors.

(B×C3H)F_1 (Virgins) foster-nursed by (C3HB×B)BC (Test series).—In this group there was a total of 355 females; 241, or 67.89 percent, developed mammary tumors at an average age of 14.98 months. In addition to the mammary tumors in the whole group were found 36 animals with leukemia, 1 with an endothelioma, 10 with hepatomas, 2 with fibrosarcomas, 1 with an osteogenic sarcoma, 6 with pulmonary tumors, 1 with a cortical adenoma of the adrenal, 2 with hemangiomas, 1 with carcinoma of the nonglandular stomach, and 2 with adenomas of the Harderian gland. Sixty-two came to necropsy without tumors.

ANALYSIS OF RESULTS

As base lines for the analysis of the results of the experiments, strain C3H can be considered as a high-tumor strain containing 100 - percent - susceptible - strain chromatin and the milk agent, while strain C57 black can be considered as a low-tumor strain containing 100-percent-resistant-strain chromatin and not having the milk agent. Hybridization of these two strains resulted in proportions of the two types of chromatin varying in amounts for the different types of hybrids, and the effects of these different proportions could be studied in relation to the action of the milk agent. These effects were manifested both in tumor incidence and tumor age.

Relationship of Tumor Incidence and Tumor Age to Proportions of Susceptible-Strain Chromatin

Before making detailed analysis of the cardinal groups of the experiment, one might analyze the data as a whole to note how the various factors, particularly the genetic factors, influence the tumor incidence and the average tumor age of the various groups. As noted in table 1, aside from varying in proportion of susceptible-strain chromatin, the different groups also varied in the type of milk agent they received which, as will be shown later, was influenced by the genetic constitution of the mother or foster mother, and furthermore there was variation in that some groups were used as breeders whereas others were kept as virgins. It therefore seemed inadvisable to attempt a statistical correlation between tumor incidence or tumor age and proportion of susceptible-strain chromatin of all groups. Comparisons were made, however, between group pairs with recognition of the variables involved in each case.

The incidence for strain C3H females, containing 100 - percent - susceptible - strain chromatin and receiving strain C3H milk agent, approaches 100 percent. That for the 14 C3H females of this experiment was 92.86 percent, which is in general accord with other published results for the strain.

Mating the C3H females to the C57 black males resulted in (C3H × B)F_1 hybrid females in which the proportion of susceptible-strain chromatin had been reduced to one-half. The milk agent, however, was derived from strain C3H. As has been found generally when high-tumor-strain females are outcrossed to low-tumor-strain males, no decrease in tumor incidence could be detected in these (C3H × B)F_1

TABLE 1.—*Mammary-tumor development in strain C3H and the various hybrid groups*

[Numerator—Number developing tumors]
[Denominator—Number dying without tumors]

Type of group	Mother	Father	Nursed by	Proportion of C3H chromatin	Number	Age in months														
						5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
C3H [1]	C3H	C3H	C3H [2]	1	14			1/0	3/0	4/0	1/0	1/1	2/0	1/0						
(C3H×B)F$_1$ [1]	C3H	B	C3H [2]	1/2	52		1/0	1/0		2/0	10/0	4/0	8/0	5/0	6/0	2/0	3/0	5/0		1/0
(C3H×B)F$_1$ [3]	C3H	B	C3H [2]	1/2	30			1/0	1/0	1/0	2/0	3/0	2/0	3/0	3/0	3/0	2/0	1/1	1/0	2/0
(C3HB×C3H)BC [1]	(C3H×B)F$_1$	C3H	(C3H×B)F$_1$ [2]	3/4	88		1/0	5/1	10/0	9/0	6/0	9/0	10/0	11/0	7/0	4/0	5/1	2/1	2/0	
(C3HB×B)BC [1]	(C3H×B) F$_1$	B	(C3H×B)F$_1$ [2]	3/4	98		0/1		0/1		1/0	3/0	4/0	6/1	5/0	8/0	2/4	5/4	4/5	5/0
(C3HB×C3H)BC×B [3]	(C3HB×C3H)BC	B	(C3HB×C3H)BC [2]	3/8	18						1/0	1/0	1/1	1/0		2/0	1/0	0/1		1/1
(C3HB×B)BC$_2$ [3]	(C3H×B)BC	B	(C3HB×B)BC [2]	1/8	45							1/1		1/0		1/2	3/0	1/1		2/3
(B×C3H)F$_1$ [3]	B	C3H	(C3HB×C3H)BC [4]	1/2	375		3/1	2/0	7/0	19/1	19/0	37/1	31/1	26/2	28/1	27/2	26/2	21/1	19/0	19/0
(B×C3H)F$_1$ [3]	B	C3H	(C3HB×B)BC [4]	1/2	355	1/0	1/0		6/0	4/1	14/2	18/1	20/4	29/4	14/0	22/0	19/1	16/1	24/4	12/1

Type of group	Mother	Father	Nursed by—	Proportion of C3H chromatin	Number	Age in months											Tumorous		Non-tumorous	
						20	21	22	23	24	25	26	27	28	29	30	Percent	Age	Percent	Age
C3H [1]	C3H	C3H	C3H [2]	1	14												92.86	9.62	7.14	11.00
(C3H×B)F$_1$ [1]	C3H	B	C3H [2]	1/2	52	1/0	1/1							0/1			96.15	12.86	3.85	24.50
(C3H×B)F$_1$ [3]	C3H	B	C3H [2]	1/2	30		1/0			0/1						1/1	90.00	14.19	10.00	23.67
(C3HB×C3H)BC [1]	(C3H×B)F$_1$	C3H	(C3H×B)F$_1$ [2]	3/4	88			1/1				0/1		0/1			93.18	11.63	6.82	19.33
(C3HB×B)BC [1]	(C3H×B)F$_1$	B	(C3H×B)F$_1$ [2]	1/4	98	2/3	2/2	2/2	1/4	1/1	0/3	1/3	1/2	1/4	0/1	0/3	55.10	16.61	44.90	21.57
(C3HB×C3H)BC×B [3]	(C3HB×C3H)BC	B	(C3HB×C3H)BC [2]	3/8	18	0/2	1/1	2/1									61.11	16.00	38.89	18.71
(C3HB×B)BC$_2$ [3]	(C3H×B)BC	B	(C3HB×B)BC [2]	1/8	45	2/3		0/3	0/2	1/5	0/6	0/7					26.67	17.17	73.33	22.39
(B×C3H)F$_1$ [3]	B	C3H	(C3HB×C3H)BC [4]	1/2	375	15/3	12/3	5/2	5/2	6/1	4/2	2/4	1/2	1/5	1/3		89.60	14.87	10.40	21.26
(B×C3H)F$_1$ [3]	B	C3H	(C3HB×B)BC [4]	1/2	355	5/3	4/2	9/3	5/4	8/6	0/5	4/6	3/9	2/32	1/21	0/4	67.89	14.98	32.11	24.71

[1] Breeders. [2] Mother. [3] Virgins. [4] Foster mother.

females from that of the high-tumor strain. The incidence for the breeding $(C3H\times B)F_1$ females was 96.15, and that for the virgins 90 percent. In this case, the difference between the breeders and virgins was insignificant. Other groups with one-half susceptible-strain chromatin were the two $(B\times C3H)F_1$ test-female groups, one of which showed a significant decrease in tumor incidence owing to variation in the milk influence as affected by the genetic constitution of the foster mother. These two are cardinal groups of the experiment and are discussed in detail later.

Backcrossing the $(C3H\times B)F_1$ females to the C3H males resulted in $(C3HB\times C3H)BC$ females that had on the average three-fourths susceptible-strain chromatin and had obtained the milk agent from $(C3H\times B)F_1$ females. The tumor incidence for this group of backcross females was 93.18 percent, and thus again no decrease in tumor incidence from that of the high-tumor strain could be detected.

In the groups with further reduction in susceptible-strain chromatin, there was a reduction in tumor incidence. It should be noted, however, that in some of these cases there was also a reduction in proportion of susceptible-strain chromatin in the mothers from which the milk agent was received. In the group of $(C3HB\times C3H)BC\times B$ virgin females that had on the average three-eighths susceptible-strain chromatin and had received $(C3HB\times C3H)BC$ milk agent, the incidence was lowered to 61.11 percent. When this incidence was compared with that of the virgin $(C3H\times B)F_1$ females that contained one-half susceptible-strain chromatin and had received C3H milk agent, it was found that the probability that the difference was due to chance alone was between 1 and 2 percent: $\chi^2=5.69$. When compared with the breeding $(C3H\times B)F_1$ females, $P<1$ percent: $\chi^2=15.28$.

The tumor incidence was lowered to 55.10 percent in the $(C3H\times B)BC$ females that contained but one-fourth susceptible-strain chromatin and had received $(C3H\times B)F_1$ milk agent. This incidence was not significantly lower than that of the small group of virgin $(C3HB\times C3H)BC\times B$ females with three-eighths susceptible-strain chromatin but was significantly lower than the incidence of the breeding $(C3H\times B)F_1$ females with one-half susceptible-strain chromatin; $\chi^2=37.99$; $P<1$ percent. The incidence of these resistant-strain backcross females, $(C3HB\times B)BC$, was significantly lower than that of the susceptible-strain backcross females, $(C3HB\times C3H)BC$, that contained three-fourth susceptible-strain chromatin and had received $(C3H\times B)F_1$ milk agent. These two groups are also cardinal groups of the experiment, for their difference constitutes a measure of the effect of variation in genetic constitution alone. They also are emphasized and discussed in detail later.

In the second backcross group of virgin females, $(C3HB\times B)BC_2$, that had received $(C3HB\times B)BC$ milk agent, the average proportion of susceptible-strain chromatin was lowered to one-eighth, and the tumor incidence was lowered to 27 percent. This incidence differed significantly from that of the virgin $(C3HB\times C3H)BC\times B$ females that had three-eighths susceptible-strain chromatin and had received $(C3HB\times C3H)BC$ milk agent: $\chi^2=6.58$; P is between 1 and 2 percent; and from that of the breeding $(C3HB\times B)BC$ females that had one-fourth susceptible-strain chromatin and had received $(C3H\times B)F_1$ milk agent; $\chi^2=100.33$; $P<1$ percent.

Thus, there was a parallel between reduction in tumor incidence and reduction in proportion of susceptible-strain chromatin of the individuals and/or proportion of susceptible-strain chromatin of the females from which the milk agent was received. Reduction of susceptible-strain chromatin in the individuals to one-half, however, did not result in a detectable reduction in tumor incidence in the groups that had received the strain C3H milk agent.

When tumor age is considered, it is found that as the proportion of susceptible-strain chromatin decreases, the average tumor age increases. As shown in table 1, the C3H females with 100-percent-susceptible-strain chromatin had an average tumor age of 9.62 months; but in contrast with the tumor-incidence results, lowering the proportion of susceptible-strain chromatin to three-fourths in the (C3HB×C3H)BC females and to one-half in the (C3H×B)F_1 females resulted in a change in the tumor age. That of the (C3HB×C3H)BC females was increased to 11.63 months, an increase with a probability of less than 1 percent; $t=3.295$. The tumor age of the (C3H×B)F_1 breeding females was further increased to 12.86 months and that of the virgins was increased to 14.19 months. If one compares the F_1 breeders to the (C3HB×C3H)BC breeders, P is found to be between 2 and 5 percent; $t=2.157$.

Decreasing the proportion of susceptible-strain chromatin to three-eighths in the (C3HB×C3H)BC×B virgin females increased the tumor age to 16 months. This, however, does not represent a significant increase over the tumor age of the virgin (C3H×B)F_1 females; $t=1.13$; P is between 20 and 30 percent; but the number of the (C3HB×C3H)BC×B females is small. Further reduction in susceptible-strain chromatin to one-fourth in the (C3HB×B)BC breeding females increased the tumor age to 16.61 months, which represents a significant increase over that of the (C3H×B)F_1 breeders; $t=5.136$; $P<1$ percent. Still further reduction to one-eighth susceptible-strain chromatin in the (C3HB×B)BC_2 virgin females increased the tumor age to 17.17 months. When compared with the (C3HB×C3H)BC×B virgin females the increase is insignificant, but the numbers are small. The increase over the tumor age of the (C3H×B)F_1 virgin females is significant; $t=2.22$; P is between 2 and 5 percent.

It is therefore evident that in general as the proportion of susceptible-strain chromatin in the individuals and/or the proportion of susceptible-strain chromatin in the mothers from which the milk agent is received is decreased, the tumor incidence is decreased and the tumor age in increased.

VALUE OF THE GENETIC INFLUENCE IN MAMMARY TUMOR DEVELOPMENT

The two groups of backcross females, (C3HB×C3H)BC and (C3HB×B)BC, were of special importance in that they provided a measure of the effect of the genetic constitution alone upon the tumor incidence. As previously pointed out, the two groups were unique in that although they differed genetically they were alike in all extrachromosomal influences. That they all came from the genetically uniform lot of (C3H×B)F_1 mothers should have contributed toward uniformity in any cytoplasmic or intra-uterine influences and particularly toward uniformity in the milk agent which they received. Additional uniformity in nongenetic factors was provided by rearing the two groups under identical conditions and using them as breeders in a comparable manner. Therefore, any difference in the tumor development of these two groups could be attributed solely to the difference in their genetic constitutions which was owing to the fact that the (C3HB×C3H)BC females had susceptible-strain fathers whereas the (C3HB×B)BC females had resistant-strain fathers.

The (C3HB×C3H)BC females with susceptible-strain fathers and thus containing three-fourths susceptible-strain chromatin had a mammary-tumor incidence of 93.18 percent, whereas the (C3HB×B)BC females with resistant-strain fathers and containing but one-fourth susceptible-strain chromatin had an incidence of

55.10 percent.[2] (See table 1.) Thus, in these groups the question whether or not the chance of a female developing a mammary tumor was about 90 percent or was but approximately 50 percent, depended entirely upon the genetic constitution of the individual. Such a difference emphasizes that the genetic constitution must be considered of primary importance in the problem of mammary tumor development in the mouse.

DEMONSTRATION OF THE GENETIC INFLUENCE ON THE PROPAGATION AND TRANSMISSION OF THE MILK AGENT

That the genetic constitution of the individual had an influence upon the ability of the individual to propagate and transmit the milk agent was demonstrated by the results of the foster-nursed groups. As pointed out in the foregoing section, the two groups of foster mothers were backcross groups which were alike in respect to all extra-chromosomal factors but different in genetic constitution. The $(C3H \times B)F_1$ mothers of both groups were of a genetically uniform lot, and this homogeneity should have contributed to uniformity in milk agent and possible intra-uterine and cytoplasmic factors which both groups of backcross females received. Since the one group of foster mothers, (C3HB×C3H)BC, had susceptible-strain C3H fathers, they had on the average three-fourths susceptible-strain chromatin and one-fourth resistant-strain chromatin, whereas the other group of foster mothers, (C3HB×B)BC, had resistant-strain C57 black fathers and therefore had but one-fourth susceptible-strain chromatin and three-fourths resistant-strain chromatin. The effect of this difference in genetic constitution on the transmission of the milk agent was apparent in the tumor development in the two groups of $(B \times C3H)F_1$ females that nursed upon these two groups of backcross foster mothers.

As shown in table 1, of the 375 $(B \times C3H)F_1$ females that nursed upon the backcross females with susceptible-strain fathers, (C3HB×C3H)BC, 336, or 89.6 percent, developed mammary tumors, whereas of the 355 $(B \times C3H)F_1$ females that nursed upon the backcross females with resistant-strain fathers, (C3HB×B)BC, only 241, or 67.89 percent, developed mammary tumors. This difference is highly significant; $\chi^2 = 51.9$; $P < 1$ percent. The difference could not be attributed to variation in the $(B \times C3H)F_1$ test females. As pointed out previously, they were a genetically uniform lot, and prior to the time they were placed with the foster mothers they had received uniform resistant-strain maternal influences and thus had not received the milk agent. Furthermore, the litters of test young had been divided between the two types of backcross foster mothers thus providing littermate controls. The difference was attributable to a difference in milk agent which the two groups of backcross females transmitted, and this difference in transmission of the milk agent was in turn owing to the difference in the genetic constitutions of the two groups of backcross females.

Thus, the probability that these $(B \times C3H)F_1$ test females would develop mammary tumors was greatly influenced by the fathers of their foster mothers. Furthermore, the tumor incidence of the test females was not only an expression of their own genetic constitution (they were all genetically susceptible) but was in part an expression of the genetic constitution of their foster mothers, an effect which would normally be transmitted from the mother to her own young through the milk.

[2] When Murray and Little (8) compared backcross females from the cross $(B \times dba)F_1$ males × dba females with backcross females from the mating $(dba \times B)F_1$ females × B males, they did not find a highly significant difference in mammary-tumor incidence between the two groups. The percentage of cancer (dba as unity) for the former group was 27.8, and for the latter 25.0. Although their situation was not exactly like the one we are reporting, from our results one would expect that their two groups also would have differed. Explanation for the difference in the two experiments is not apparent.

A more detailed analysis was made of the transmission of the milk agent by the individual backcross females. In table 2, the females of the two backcross groups are listed by number, separated according to whether or not they developed a mammary tumor, and with each female is indicated the age at which each of her test $(B\times C3H)F_1$ fostered females developed a mammary tumor or came to necropsy without having developed a mammary tumor. In viewing these data, it must be borne in mind that owing to the segregation of genes in the production of the backcross generations, both backcross groups were genetically variable, the $(C3HB\times C3H)BC$ females having from 50 to 100 percent C3H chromatin, with an average of 75 percent, and the $(C3HB\times B)BC$ females having from 0 to 50 percent C3H chromatin, with an average of 25 percent. In this respect, the backcross foster mothers differed from the $(B\times C3H)F_1$ fostered young which were genetically uniform.

The milk agent was variable. The situation, as illustrated by the data in table 2, was not one in which the backcross females either did or did not transmit the agent, but their transmission of the agent varied in degrees from highly effective transmission, indicated by all the test females developing tumors at an early age, to extremely ineffective or no transmission of the agent, indicated by all the test females dying nontumorous at old age. What this variation is, however, has not been determined. It may be a variation in concentration or virulence of the agent or, in reference to a latent virus, it might conceivably be a variation in mutation rate. Table 3 summarizes the data in reference to this variation.

Since the difference in transmission of the milk agent by the two groups of backcross females is attributable to a genetic difference, it follows that this variation within the groups is likewise attributable in part at least to genetic variation resulting from the segregation of genes. This genetic variation is more apparent in the resistant-strain

TABLE 2.—*Test of effect of genetic constitution on propagation and transmission of the milk agent* [1]

Susceptible strain backcross tumorous females (C3HB×C3H)BC		Foster-nursed females $(B\times C3H)F_1$	
Female No.	Age tumor appeared.	Age females had tumors	Age females died nontumorous
	Months	*Months*	*Months*
7012	17	9, 9, 12, 10, 15	
7016	13	19, 22, 17, 10	21.
7018	12	8, 11, 11, 14, 12	
7019	11	24, 16, 17, 18, 14	
7020	13	19, 13, 10, 16	
7027	13	14, 11, 8, 13, 12	
7029	13	12, 15, 18, 10, 9	
7033	15	20, 14, 14, 17, 11	
7035	14	17, 15, 21, 24, 12	
7036	12	13, 18, 16, 17, 10	
7044	9	21, 21, 16, 16, 16	
7045	9	19, 15, 15	6, 29.
7046	8	16, 11, 11, 12, 12	
7047	8	15, 14, 12, 18	
7052	8	12, 16, 17, 12	24.
7053	7	13, 18, 20, 21	
7054	10	12, 16, 12	26, 26.
7079	7	10, 13, 14, 11, 16	
7080	7	11, 16, 16, 13	28.
7087	7	15, 23, 9	9, 20.
7088	8	14, 24, 16, 18	
7098	16	15, 14, 17, 19, 19	
7101	16	11, 18, 23, 16, 21	
7118	13	16, 19, 17	23, 26.
7119	11	6, 16, 14, 14	16.
7120	16	17, 11, 16, 10, 13	
7122	16	11, 12, 15, 11, 7	
7128	14	14, 12, 21, 21, 19	
7129	17	20, 15, 13, 11, 13	
7131	12	9, 9, 20, 16	
7132	13	15, 22, 20, 20	29.
7133	16	16, 14, 28, 14, 15	
7184	13	16, 18, 17, 13, 7	
7187	8	17, 13, 10	
7188	10	13, 15, 13	
7189	14	12, 10, 15, 11	
7245	22	20, 11, 11, 24	
7246	8	15, 10, 18, 8, 14	
7247	9	12, 14, 11, 15, 18	
7271	11	26, 18, 11, 29	
7273	9	19, 11, 9, 13, 8	
7274	11	16, 12, 18, 11, 11	
7320	15	13, 13, 19, 9, 17	
7321	11	15, 14, 10, 19, 10	
7322	15	11, 6, 17	
7323	11	20, 10, 14, 18, 12	
7353	12	12, 12, 25, 21	11.
7354	13	12, 14	22.
7355	12	25, 19, 13, 10, 20	
7359	6	11, 21, 10, 23	
7360	14	21, 11, 21, 14	
7361	15	15, 20, 9	15, 17.
7362	18	19, 17, 12, 17, 17	
7363	14	22, 13, 12, 14	27.
7364	13	20, 19, 11, 9, 16	
7368	11	15, 8, 10, 23, 14	
7380	9	9, 11	15.
7381	11	13, 12, 13, 10, 19	
7382	14	10, 9, 9, 11	
7384	14	13, 13, 16	
7835	18	10, 11, 20, 9, 6	
7871	10	9, 19, 14	23.
7873	9	11, 14, 12	20, 13.
7941	8	12, 15, 21	13, 21.
7942	12	19, 12, 12, 27	
7995	10	8, 9, 15, 18	
7996	7	14, 17, 18, 12, 17	
7997	13	9, 25, 15, 12, 13	
7998	8	18, 11, 19, 14, 11	
7999	13	11, 17, 18, 15	

Footnote at end of table.

TABLE 2.—*Test of effect of genetic constitution on propagation and transmission of the milk agent*[1]—Continued

Susceptible strain backcross nontumorous females (C3HB×C3H)BC		Foster-nursed females (B×C3H)F1	
Female No.	Age died nontumorous	Age females had tumors	Age females died nontumorous
	Months	*Months*	*Months*
7090	16	16, 23, 22, 14	
7099	7	24, 11, 22, 24	28
7130	26	20, 14, 25, 20	12.
7383	28	20	16, 28, 26, 22.
7872	17	18	28, 28, 20, 20.
7875	22		27, 25, 25, 21.

Resistant strain backcross tumorous females, (C3HB×B)BC		Foster-nursed females (B×C3H)F1	
Female No.	Age tumor appeared	Age females had tumors	Age females died nontumorous
	Months	*Months*	*Months*
7199	15	13, 16, 17, 17	
7205	15	11, 18, 13, 15	18.
7206	13	16, 14, 15, 8, 11	
7219	17	12, 10, 15	
7221	15	9, 15, 15	16, 24.
7227	17	21, 28, 19, 10	13.
7228	15	29, 12, 12, 18	20.
7229	15	17, 14, 22, 11	
7230	13	9, 17, 14	12.
7256	24	12, 27, 22, 24, 11	
7265	26	14, 20	12.
7301	18	16, 11, 13, 13, 8	
7303	14	11, 11, 13, 19	25.
7306	14	16, 8, 12, 12	
7325	10	12, 15, 11, 10, 10	
7326	12	11, 19, 13, 18, 17	
7327	27	6, 19, 20, 13, 15	
7328	13	9, 10, 10	
7329	14	13, 13, 13, 10	
7330	23	10, 11, 19, 11, 14	
7335	19	18, 13, 13, 17	29.
7338	17	11, 16, 14, 17	21.
7340	12	24, 22, 18, 14, 11	
7346	13	24, 18, 16, 15, 15	
7373	12	12, 12, 15, 18, 14	
7374	16	13, 13, 13, 13	
7375	13	23, 18	25.
7377	20	17, 15, 15	
7844	15	14, 16, 18	17.
7846	13	8, 15, 20	
7851	14	14	
7852	15	16, 16, 12, 21	12.
7854	19	18, 5, 19	
7855	19	15, 17, 14, 11, 19	
7856	17	9, 12, 15, 8, 12	
7862	22	15, 22, 16	
7863	20	21, 12, 20, 23, 18	
7864	11	11, 17, 13, 13	29.
7865	19	17	11.

TABLE 2.—*Test of effect of genetic constitution on propagation and transmission of the milk agent* [1]—Continued

Resistant strain backcross nontumorous females, (C3HB×B)BC		Foster-nursed females (B×C3H)F1	
Female No.	Age died nontumorous	Age females had tumors	Age females died nontumorous
	Months	*Months*	*Months*
7061	18	19, 20, 22	9.
7062	25	22, 14	27, 27, 27.
7063	13	24, 28	10, 27.
7064	21	18, 13, 13, 16	
7065	27	13	29, 27, 27, 28.
7072	18	24	29, 29.
7073	20	27, 12	29, 28, 23.
7109	16	18, 15, 12, 15	
7112	18	14, 19	18, 26.
7194	17	8, 16, 10, 19	
7195	23		28, 28, 28, 27, 28.
7196	23	18, 22	28, 24, 25.
7197	18		24, 28, 28, 20, 28.
7198	8	17, 16, 11	
7209	16	13, 13, 17, 17	23.
7212	20		28, 28, 27, 28, 28.
7213	22	23, 26, 23	22, 18.
7255	30	12, 16, 22, 22, 13	
7261	17		29, 29, 28, 25, 29.
7264	16		23, 30, 21, 29, 26.
7283	28	13, 26, 24	22, 10.
7292	26	12, 13, 26, 18	24.
7293	26	17, 18, 15, 16	28.
7294	16		20, 18, 13, 13, 12.
7302	23		28, 28, 26, 29.
7304	30	15, 12, 10	29.
7305	24	27, 14	30, 26, 29.
7336	23		27, 22, 29, 26, 29.
7337	22	18, 18, 23	28.
7345	28	18, 12	28, 28, 28.
7376	17	17	24, 19.
7845	20	13, 10, 13, 10	13.
7850	18	19, 16, 16	28.
7866	21	10, 16, 18, 12, 11	
7991	29	16, 19, 18	29, 24.

[1] At weaning time, 2 litters for testing the (C3HB×C3H)BC females and 2 for testing the (C3HB×B)BC females each contained 1 animal that might have nursed upon 2 different females of the respective group, and thus these litters had to be omitted from these individual tests although included in the tests of each group as a whole in table 1.

backcross (C3HB×B)BC females than in the susceptible-strain backcross (C3HB×C3H)BC females, which is to be expected in view of the greater genetic variation in tumor development in the resistant-strain backcross females. The variation displayed by the susceptible-strain backcross females was owing primarily to three individuals (table 2, Nos. 7383, 7872, 7875) all

TABLE 3.—*Variation in the milk agent transmitted by the two types of backcross females*

Type of foster mother	Number with given percent of tumorous fostered females										
	0	20	25	33⅓	40	50	60	66⅔	75	80	100
(C3HB×C3H)BC:											
Tumorous							7	2	1	7	53
Nontumorous	1	2								2	1
Total	1	2					7	2	1	9	54
(C3HB×B)BC:											
Tumorous						1	1	2	2	8	25
Nontumorous	8	1		2	5	2	3		4	4	6
Total	8	1		2	5	3	4	2	6	12	31

or most of whose fostered young died nontumorous at advanced age. Two of these backcross females were derived from a nontumorous F_1 mother, indicating that the milk agent may have been inactivated or reduced in the preceding generation in this case.

While there is evidence that genetic segregation influences the propagation and transmission of the milk agent, there is no indication that but a single gene is involved. If such were the case, one would expect half of these resistant-strain backcross females to have transmitted the agent and the other half not to have transmitted it. The continuous variation suggests the action of multiple factors, thus falling in line with many other cancer-inheritance studies in the mouse (*17–23*).

The transmission of the agent was more effective by the backcross females that developed mammary tumors than by those that did not. The data are summarized in table 4. The 323 (B×C3H)F_1 females that nursed upon (C3HB×C3H)BC females which later developed mammary tumors gave an incidence of 93.2 percent, while the 28 that nursed upon nontumorous (C3HB×C3H)BC females had a mammary-tumor incidence of only 50 percent. Similarly, an incidence of 90 percent was obtained for the 165 (B×C3H)F_1 females nursed by the (C3HB×B)BC females which later developed mammary tumors as compared with an incidence of only 49.7 percent obtained for the 159 (B×C3H)F_1 females nursed by the nontumorous (C3HB×B)BC females. It is noteworthy, however, that although the nontumorous females did not transmit the agent as effectively as did those that later developed tumors, they did transmit the agent so that half of the fostered young developed tumors, and this proportion is far from insignificant.

A possible explanation in part for the failure of many of the backcross females to develop mammary tumors, although they did propagate and trans-

TABLE 4.—*Tumorous backcross females compared with nontumorous in their ability to transmit the milk agent*

Foster mothers	Fostered females					
	Test females	Number	Tumorous		Nontumorous	
			Percent	Average age	Percent	Average age
				Months		*Months*
C3HB×C3H)BC Tumorous	(B×C3H)F_1	323	93.2	14.7	6.8	20.0
C3HB×C3H)BC Nontumorous	(B×C3H)F_1	28	50.0	19.5	50.0	23.9
C3HB×B)BC Tumorous	(B×C3H)F_1	165	90.9	14.9	9.1	18.9
C3HB×B)BC Nontumorous	(B×C3H)F_1	159	49.7	16.8	50.3	25.0

mit the milk agent, is that there are two sets of genetic factors involved, one determining whether or not the agent will be propagated and the second influencing the susceptibility of the mammary-tissue cell to the carcinogenic action of the agent. In order for a tumor to appear, both sets of factors would have to be present; and thus any mouse with a tumor would be expected to be able to transmit the agent, as the results show. According to this possible explanation, a mouse could fail to inherit the genes making the mammary cells susceptible although it did inherit the genes favorable to the propagation of the agent. Such a mouse would be able to transmit the agent but would not develop a tumor. It is recognized, however, that a part of the explanation can be in nongenetic factors. In the genetically homogeneous, high-tumor strains, a few females do not develop tumors although they do transmit the agent, and this must be explained on the basis of nongenetic factors alone. Genes acting through the hormonal mechanism would not enter into this consideration of the backcross females since all were used as breeders.

The data in tables 2 and 3 show that the nontumorous backcross females which transmitted the agent did not transmit it so effectively on the average as did the tumorous females. This was indicated by the percentage of fostered test females developing tumors. These results suggest that some of the factors controlling susceptibility to the agent are likewise related to the propagation and transmission of the agent.

In these groups of test $(B\times C3H)F_1$ females, there was a difference in average tumor age, those fostered by the nontumorous backcross females having a higher tumor age than did those fostered by the females that later developed tumors. This difference cannot be owing to a difference in the genetic constitution of the individual animals, but it may be attributed in part at least to differences in extrachromosomal factors.

BREEDING TEST OF BACKCROSS SEGREGANTS

The second backcross to the resistant strain, $(C3HB\times B)BC_2$, provides a breeding test of the genotype of the tumorous and nontumorous first-backcross segregants, a type of test recognized as essential in establishing proof of single-factor inheritance as distinguished from multiple-factor inheritance.

Assuming a character controlled by a single dominant factor (A) with (a) as the recessive allele, a cross between the homozygous dominant (AA), with the character, and the homozygous recessive (aa), without the character, results in all the F_1's showing the character but heterozygous (Aa). The F_1 (Aa) backcross to the homozygous recessive parent (aa) results in half the progeny's showing the character and of the genotype (Aa) and half not showing the character (aa). If the segregants with the character, (Aa), are in turn backcrossed (the second backcross) to the homozygous recessive, the expected ratio in turn will be like that from the first backcross, i. e., half heterozygous (Aa) and showing the character, and half homozygous for the recessive allele (aa). Furthermore, if the first-backcross segregants not showing the character and of the genotype (aa) are in turn backcrossed to the homozygous recessive (aa) parent, the expectancy would be 100 percent (aa) and not displaying the character. The fulfillment of these expected ratios in the second backcross is considered as conclusive evidence essential for the verification of F_1, F_2, and backcross ratios indicating single-factor inheritance.

In this experiment, 33 of the 45 second-backcross females were from tumorous first-backcross females and 12 were from nontumorous first-backcross females. Of the 33 from the tumorous first-backcross females, 11, or 33.33 percent, developed tumors, which differs from the 55.10 percent tumor incidence in the first-backcross generation; $\chi^2=4.68$; $P<5$ percent. Of the 12

from nontumorous first-backcross females 1 developed a mammary tumor, although her mother died nontumorous at 30 months. That the progeny of the tumorous mothers had the higher incidence of mammary tumors is to be expected with a genetic influence. A single factor, however, is not indicated, and thus the data although not extensive fall in line with the other results of the experiment representing multiple-factor inheritance.

DISCUSSION

The problem of tumor development is primarily a problem in cell physiology. The role of the genes can be emphasized only with a full recognition that the cytoplasm is the substratum on which the genes act and the medium in which they exist and have their function. Wright (*24*) pointed out that the usual and most probable view of cellular differentiation is that it is cytoplasmic. The interaction of nuclear and cytoplasmic products with the environment leads to the elaboration of plasmagenes, determiners within the cytoplasm and transmitted to daughter cells by cytoplasmic heredity.

Of primary importance in the development of mammary tumors in the mouse is the milk agent, which, although usually transmitted from parent to offspring through the milk, can be viewed as a substance within the cytoplasm transmitted to daughter cells by cytoplasmic heredity and instrumental in the differentiation from normal to malignant cells. By adding to this concept the role of the nucleus in the propagation of the milk agent, as was demonstrated by the experiments reported herein, one formulates a picture in many respects like the nuclear-cytoplasm relationship concept of cell differentiation.

Recent discussions by Darlington (*25*) and Haddow (*26*) illustrate an awakening to the importance of gene-cytoplasm interrelation in tumor development. Emphasis is placed on the increasingly recognized role of plasmagenes in animals, plastogenes in plants, and viruses as cytoplasmic inherited determiners related to the genes and playing an active part in cell differentiation, a subject paramount in cancer. Basis for this line of thought was in experimental work such as that of Sonneborn (*27*, *28*) who described a character in *Paramecium* which is dependent upon both a cytoplasmic substance and a dominant gene: that of Rhoades (*29*) who described a gene-induced transmissible plastid difference, and that of Woods and DuBuy (*30*, *31*) whose work suggests the possibility of origin relationship of certain viruses to the cytoplasmic elements, mitochondria.

To illustrate how the mamary-tumor problem fits this gene-cytoplasm relationship concept, let us compare the mamary-tumor situation with that reported by Sonneborn. In studying the character "killer" in *Paramecium aurelia*, Sonneborn found that manifestation of the character was dependent upon two factors, one cytoplasmic and the other chromosomal, which from mating tests proved to be a single dominant gene that he designated as (*K*). Sensitive (*KK*) clones existed which, without the cytoplasmic factor, did not manifest the character but became killer clones when the cytoplasmic factor was introduced, and thereafter the character was transmitted to future generations. When the cytoplasmic factor was introduced into clones homozygous for the recessive allele (*kk*), however, the character was not manifest, nor was the cytoplasmic factor perpetuated. Thus, the phenotype was controlled by a determiner which was outside the nucleus and which was dependent on a dominant gene for its propagation.

Mammary-tumor development in the mouse is governed largely by a cytoplasmic factor, the milk agent, which in turn is under the control of the nucleus. With genetic variation owing to the segregation of genes, the milk agent also varies, as is observed by the effectiveness with which the female will transmit it to susceptible young. In the first resistant-strain backcross gen-

eration, this variation ranged from females which transmitted the agent so that all the test young developed tumors to those which either did not transmit the agent at all or transmitted it only in a slight degree, for none of their test females developed tumors. Thus, as with Sonneborn's *Paramecium* character, the propagation and transmission of the milk agent was dependent upon the genotype.

Further comparison can be made between the inbred mouse strains and Sonneborn's *Paramecium* strains. High-tumor strains, such as C3H, A, and dba, are high-tumor strains because they have the milk agent which can be propagated since each strain has a suitable genotype. When Bittner (*32*, *33*) removed the milk agent from strain A mice by foster-nursing them on a low-tumor strain, the resultant strain Ax was a low-tumor strain although the genotype was unaltered. By the reintroduction of the milk agent, however, the strain again became a high-tumor strain. When Murray and Little (*8*) removed the suitable genotype by repeatedly backcrossing to the resistant-strain male from the original cross high-tumor-strain dba female × low-tumor-strain C57 black male, they found that not only were there no tumors developed but also that they had eliminated the milk agent.

Strain C57 black appears to be a low-tumor strain because it lacks the suitable genotype and therefore lacks the milk agent. Andervont [3] found that strain C57 black females that nursed upon strain C3H foster mothers transmitted the milk agent to susceptible young that develop tumors, but later generation C57 black females did not transmit the agent to susceptible young. Fekete and Little (*34*), however, obtained a relatively high mammary-tumor incidence through three generations of progeny of C57 black females that were developed from fertilized ova transferred to dba females. Introduction of suitable chromosomal factors alone by mating C57 black females to C3H males, or as Murray and Little did through repeated backcrosses to dba males, does not result in tumor development, for the mere introduction of the suitable genes does not give rise to the milk agent. Exception to this is the case reported by Bittner (*33*) in which the agent arose in a low-tumor, fostered, strain A female. The introduction of both the milk agent and suitable chromosomal factors, as by mating C57 black males to C3H females, gives rise to a high-tumor hybrid generation.

It now appears to be clearly established that strain C is a low-tumor strain not because it lacks the suitable genotype but because it lacks only the milk agent. Andervont (*35*) has found that the introduction of the milk agent into strain C by foster-nursing them by strain C3H females resulted in a high-tumor line that to date has remained a high-tumor line through 12 generations.

Thus, just as these other characters result from cytoplasmic inherited determiners which in turn are dependent upon certain genes, mammary tumor of the mouse can result from the milk agent, which has many characteristics in common with viruses but can also be considered as a cytoplasmic agent, and this milk agent is in turn dependent upon chromosomal factors.

Complexity is added to the problem by the variations in the transmission of the agent. Just as Sonneborn found some variation in the cytoplasmic factor between homozygous (*KK*) and heterozygous (*Kk*) so is there variation in milk agent in animals of different genotypes. Furthermore, just as Sonneborn found that several generations (several fissions) were required for the gene effect to become manifest so also can this variation in the milk agent transmitted have some influence upon tumor development regardless of the genotype of the animal receiving the agent. The test females of the experiments reported herein all have identical genotypes, but the different groups varied greatly in tumor development be-

[3] Andervont, H. B.: Unpublished data.

cause of the variation in the milk agent which they received.

In the light of the variation in tumor development resulting from the variation in transmission of the agent, which in turn is owing to the variation of the genotype of the mothers or foster mothers, this phase of the problem becomes one in which primary gene action in one generation is manifest in the following generation that may even be a fostered generation. The development of the tumor is influenced not only by the genetic constitution of the individual but also by that of the mother or foster mother through the transmission of the milk agent. This influence would come under the general heading of influence of the genetic constitution of the stock. Such genic influences can presumably be transmitted in a number of ways, such as through the milk (as in the present case), through the uterine wall, or through the cytoplasm of the egg. A classic case of the third possibility is the direction of the coil of the snail, *Lymnaea peregra*. Boycott et al. (*36*) found that the direction of the spiral was not a manifestation of the genotype of the individual but of the genotype of the mother. This finding indicated that the direction of the coil was determined in the cytoplasm by the genotype of the egg prior to reduction division and fertilization. The example further emphasizes the role of the cytoplasm in transmitting genic influences to future cell generations.

The aspect of the mammary-tumor problem relative to the genetic control of the transmission of the milk agent is comparable with situations regarding many plant viruses in which there is a genetic variation in the ability of the vector to transmit the virus. Black (*37*) produced two lines of clover leafhoppers, one of which was "active" in transmitting potato yellow dwarf virus and the other "inactive." Of the active race, 80 percent were infective, while of the inactive race only 2 percent were. Of their F_1 hybrids 30 percent were infective. Storey (*38*) bred two races of *Cicadulina mbila*, one of which could transmit maize-streak virus and the other could not. Other examples could be cited. Such problems involve the genetics of the host, and that of the vector.

In the mouse-mammary-tumor problem, the genetics of the vector and that of the host governing susceptibility to the agent appear to be represented as two sets of factors in the one organism, the mouse. One set of factors influences the propagation and transmission of the agent while the other set determines the susceptibility of the mammary-tissue cells to the carcinogenic action of the agent. It could be assumed that mice which developed tumors would have both sets of factors and would therefore be able to transmit the agent, whereas some mice might lack the susceptibility factors and would not develop tumors although they did have the factors necessary for the transmission of the agent. The data of the experiment reported herein support such a hypothesis. That the two sets have some factors in common, however, is evidenced by the fact that the nontumorous backcross females which did transmit the agent transmitted it less effectively on the average than did the tumorous backcross females all of which transmitted the agent.

This discussion in no way minimizes the importance of the milk agent, or of the value that would be derived from the discovery of a comparable agent in relation to human breast cancer. The experiments, however, do stress the importance of the genetic factors and of the intimate relationship of the genetic factors to the milk agent. If solely by altering the paternal genes one can vary the chance of a female's developing a tumor from above 90 percent to approximately 50 percent, as was done in the two backcross groups, the genetic effect cannot be minimized. Furthermore, if, in a single generation, segregation of genes results in variation in ability to transmit the agent from females which effectively trans-

mit the agent to 100 percent of their fostered young to those which do not effectively transmit the agent to any of their fostered young, the intimate interrelation of the genes to the milk agent likewise cannot be minimized.

The experiments throw light on some of the problems that might be encountered in the discovery and study of an agent similar to the milk agent in a genetically heterogeneous human population. It is reasonable to expect that such an agent would probably be closely related to the genetic constitution of the individual not only in that the genetic factors would influence whether or not the agent succeeded in exciting tumor formation but would also influence the transmission of the agent. While women who develop tumors could be expected to transmit the agent better than nontumorous women, it could be expected that certain nontumorous women would also transmit the agent. The extreme genetic variability in the human population would be expected to affect extreme variability in the transmission of the agent: but since no conscious accumulation of favorable genes has been made, one would expect very few, if any, individuals to transmit the agent in a degree comparable with high-tumor, inbred, mouse strains. By affecting the transmission of the agent, the genetic constitution of the mother, as well as that of the individual, could have a great effect on influencing the probability of the individual's developing breast tumor.

Because of these possible complicating genic influences one does not wonder that such an agent has not been identified in the human being. It is reasonable to expect, however, that the breast-tumor problem in the human species like that of the mouse may be a problem in cell physiology involving an interrelation between genic action and a cytoplasmic determiner similar to the milk agent. This concept offers a hope that such factors can be identified.

SUMMARY

The genetic constitution of the mouse has an influence on its ability to propagate and transmit the milk agent. Susceptible-strain backcross females with an average of 75 percent susceptible-strain chromatin transmitted the agent more effectively to the test females than did resistant-strain backcross females with an average of 25 percent susceptible-strain chromatin, although both backcross groups had received identical milk agent and other maternal influences.

In the resistant-strain backcross group, it was further evident that segregation of genes resulted in a variation in degree of transmission of the agent from females which transmitted the agent so effectively that all their test females developed tumors at an early age to those which did not transmit the agent or transmitted it so ineffectively that none of their test females developed tumors. This variation in degree suggested the segregation of more than one pair of genes influencing the propagation and transmission of the agent.

The results indicated that there are two sets of genetic factors, aside from those acting through the hormonal mechanism, that influence mammary-tumor development. One set determines the susceptibility of the mammary-tissue cell to the hormonal and milk-agent stimuli, and the other the propagation of the milk agent. All tumorous backcross females were able to transmit the agent, whereas only a part of the nontumorous females transmitted the agent while others did not. That these two sets had one or more factors in common was indicated by the fact that the nontumorous backcross females which did transmit the agent transmitted it less effectively than did the tumorous backcross females.

By comparing the tumor incidences of the two groups of backcross females that had all extrachromosomal factors uniform, it was found that whether the

chance of a female's developing mammary tumor was above 90 percent or was only approximately 50 percent was determined by genetic factors alone.

Results of the various hybrid groups indicated that as the proportion of susceptible-strain chromatin in the individual and/or the proportion of susceptible-strain chromatin in the mothers from which the milk agent is received is decreased, the tumor incidence is decreased and the tumor age is increased.

The results of a breeding test of the resistant-strain backcross tumorous and nontumorous segregants were such as would be expected with multiple-factor inheritance.

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DATA ON CANCER THERAPY

AT the Gibson Island Cancer Symposium in August 1944, a suggestion was made that some one compile and publish all the data available relative to cancer therapy. Such a survey might be expected to aid other workers in planning systematic studies in this field of cancer research. The National Cancer Institute has offered to undertake this work.

All types of therapy except surgery and irradiation will be covered. Negative and positive results of the treatment of spontaneous, transplanted and induced experimental tumors, and of clinical cases, will be included. It has been found that negative results which have been obtained have not always been published. The data will be classified and tabulated in a simple manner similar to that in Hartwell's "Survey of Compounds Which Have Been Tested for Carcinogenic Activity," National Cancer Institute, 1941, and will include the name of the agent, the number of animals or patients treated, the dosage, route of therapy, number of treatments, the authors' conclusions regarding a beneficial effect, the percentage of complete regressions if obtained and the reference.

It would be appreciated if all available reprints of published work in this field or any unpublished results which may be used are sent to the undersigned.

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